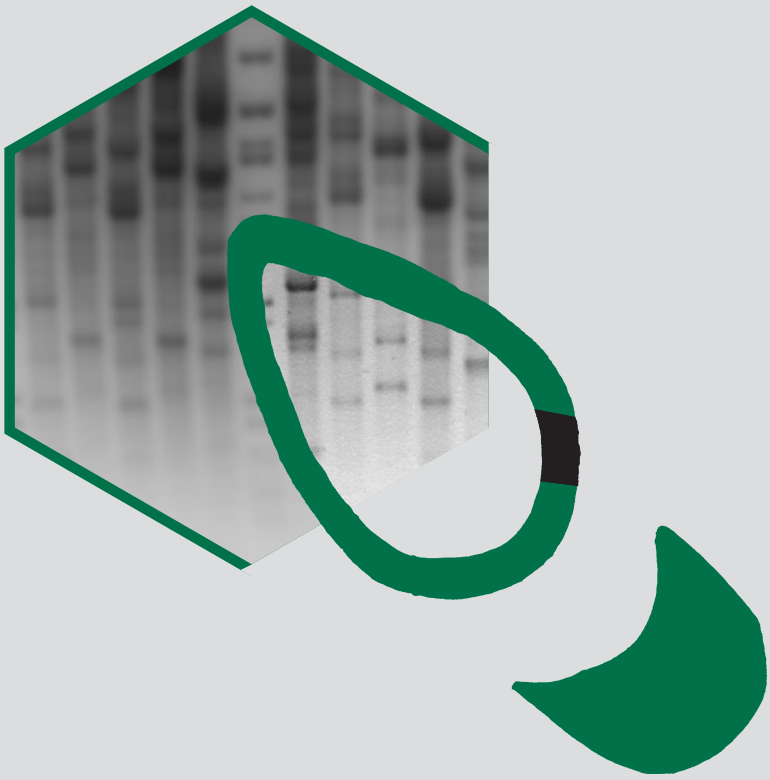

Molecular Biomonitoring during Rhizoremediation of Oil-Contaminated Soil

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Dissertationes bioscientiarum molecularium Universitatis Helsingiensis in Viikki

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Academic Dissertation in Microbiology

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Helsinki 2006

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Next page: Dry translation from not so dry Northern Karelian dialect:
Have I the face to talk to You? I have almost died and tipped over in this company, and it would be lighter to walk if I got more money.

Ilikijääkö Teile huastoo? Mie oon melekee kuolta keikahtana tiällä virmassa ja miusta ois keppiimp käyvä, jos sais lissää rahhoo.

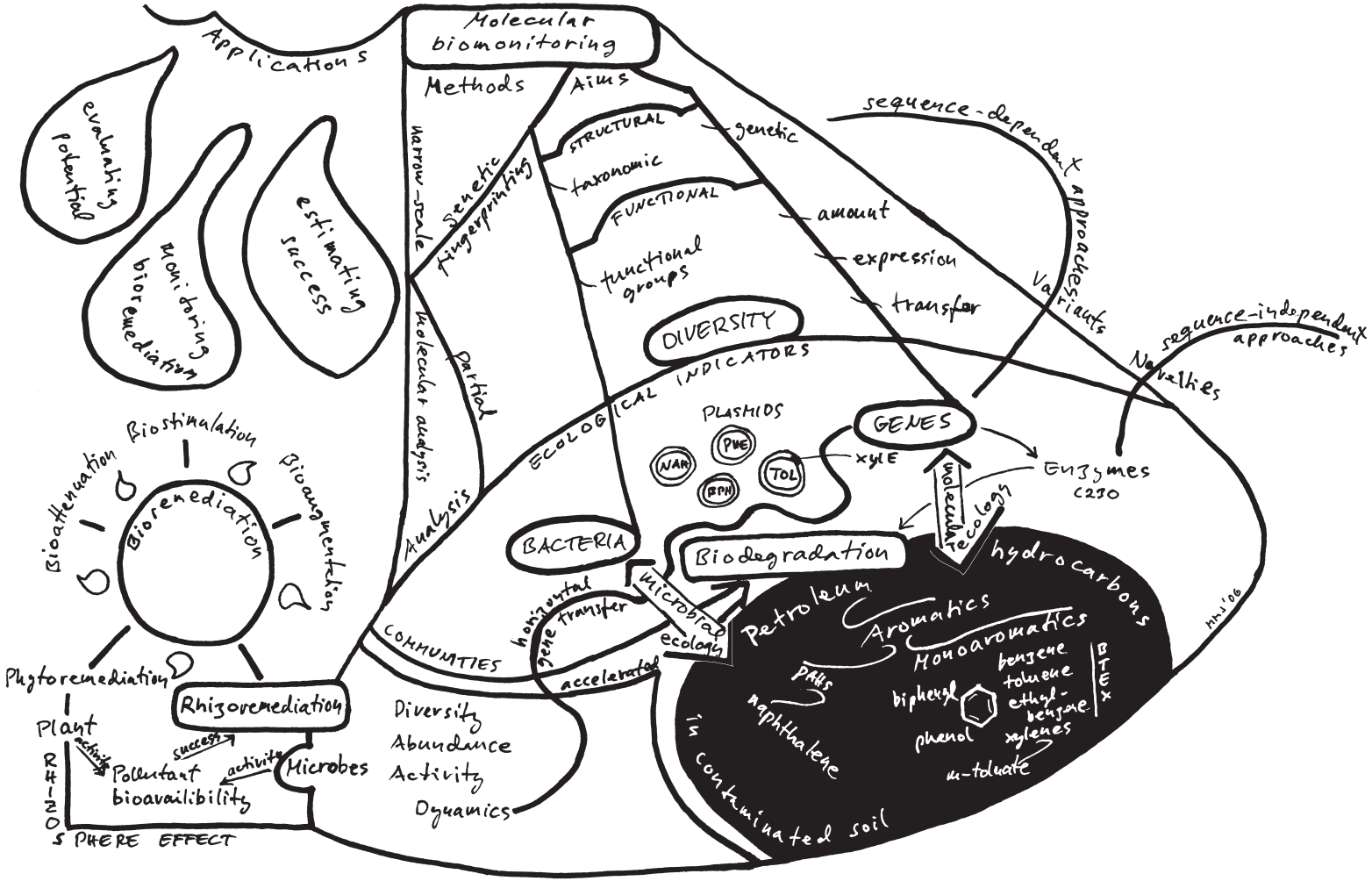
Table of contents

Preface

This book for the degree of Doctor in Philosophy is written in a typical form of PhD Theses in natural sciences. It consists of original, peer-reviewed articles published in international scientific journals and a summary of the whole work. Having pedagogical background, I found it interesting to write the section 'Introduction' (a literature review) in such a way that it might benefit university lecturers, other teachers and students when dealing with the progress in the field of environmental biotechnology and molecular ecology. Bioremediation, oil-degrading bacteria and their catabolic genes, and molecular biomonitoring methods will be the future hot topics also among the general public. In this context, it has been challenging to clarify the essential terms (marked with **bold** letters) and organize the content in a meaningful way. For visually oriented persons and in order to quickly extract the main content in the spirit of 'mind mapping', central words in each paragraph are marked with SMALL CAPS.

I have been lucky to see continuity in my work: my Master's Thesis at the Faculty of Biosciences touched the two-component regulatory systems, which I found useful, when I proceeded in my scientific carrier and entered the nitrogen-fixation group at the Faculty of Agriculture and Forestry. During this work, in the era of stepping 'from sequences to function and regulation', thoughts about new teaching entities and how to combine research and scientific teaching and learning in a more efficient way have occupied my mind. We should not loose the chances for Bio-Finland. Through specializing in bioremediation in the *Galega* rhizosphere, it has become obvious to me that interactive signalling between the plant and some rhizosphere bacteria and plasmid dynamics, i.e. gene transfer, between bacteria might also be essential in the rhizoremediation of oil-contaminated soils. I invite the reader of this thesis to share some details behind the big picture still waiting to be discovered. Feedback on this book is welcomed directly ;) or via email (minna.m.jussila@hotmail.com).

Fig. ;) . Personal mind map of this thesis to inspire the reader's own interpretation and novel thoughts. (on page 7)


Applications
evaluating potential
monitoring bioremediation
estimating success
Molecular biomonitoring
Methods
Aims
narrow-scale
Genetic fingerprinting
partial
Molecular analysis
Analysis
STRUCTURAL
genetic
taxonomic
FUNCTIONAL
amount
expression
transfer
functional groups
DIVERSITY
ECOLOGICAL INDICATORS
sequence-dependent approaches
Variants
sequence-independent approaches
Newalkies
PLASMIDS
NAH
PHE
RPH
TOL
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GENES
Enzymes
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molecular ecology
BACTERIA
Biodegradation
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Aromatics
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xylenes
BTEX
m-toluate
in contaminated soil
Bioremediation
Biostimulation
Bioaugmentation
Bioattenuation
Phytoremediation
Rhizoremediation
Plant
activity
success
Pollutant bioavailability
activity
Microbes
RHIZOSPHERE EFFECT
Diversity
Abundance
Activity
Dynamics
mm'06

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. The original publications were reprinted with the publisher's kind permission. *, corresponding author.

I Suominen, L.*, **Jussila, M.M.**, Mäkeläinen, K., Romantschuk, M. & Lindström, K. 2000. Evaluation of the *Galega - Rhizobium galegae* system for the bioremediation of oil-contaminated soil. Environmental Pollution 107, 239-244.

II Lindström, K.*, **Jussila, M.M.**, Hintsa, H., Kaksonen, A., Mokelke, L., Mäkeläinen, K., Pitkäjärvi, J. & Suominen, L. 2003. Potential of the *Galega - Rhizobium galegae* system for bioremediation of oil-contaminated soil. Food Technology and Biotechnology 41, 11-16.

III **Jussila, M.M.***, Jurgens, G., Lindström, K. & Suominen, L. 2006. Genetic diversity of culturable bacteria in oil-contaminated rhizosphere of *Galega orientalis*. Environmental Pollution 139, 244-257.

IV Kaksonen, A.H., **Jussila, M.M.***, Lindström, K. & Suominen, L. 2006. Rhizosphere effect of *Galega orientalis* in oil-contaminated soil. Soil Biology & Biochemistry 38, 817-827.

V **Jussila, M.M.***, Zhao, J., Suominen, L. & Lindström, K. 2006. TOL plasmid transfer during bacterial conjugation *in vitro* and rhizoremediation of oil compounds *in vivo*. Environmental Pollution, doi: 10.1016/j.envpol.2006.07.012 (1-15), in press.

The author's contribution

Paper I

The experiments were planned together. Minna M. Jussila and Katri Mäkeläinen contributed to the greenhouse experiment. Minna M. Jussila contributed to the supervision in the laboratory, and the analysis and interpretation of the results. Leena Suominen analyzed and interpreted the results, wrote the paper, and carried out the correspondence. Martin Romantschuk acted as the coordinator of two bioremediation projects at the University of Helsinki. Kristina Lindström was the project leader and supervisor of the Ph.D. studies of Minna M. Jussila.

Paper II

Kristina Lindström wrote the review of the bioremediation project and was the corresponding author of the article. She was the project leader and supervisor of the Ph.D. studies of Minna M. Jussila. Minna M. Jussila conducted the experiments for her Ph.D. thesis, contributed to the supervision of the master students and the writing of the paper. Hannamari Hintsa, Anna Kaksonen, Lena Mokelke and Katri Mäkeläinen did their master's theses in the project. Jyrki Pitkäjärvi acted as an expert on genetically modified organisms. Leena Suominen acted as an expert on the greenhouse and field experiments.

Paper III

Minna M. Jussila planned and conducted the experiments, analyzed and interpreted the results, and wrote the paper. German Jurgens supervised Minna M. Jussila in the use of the ARB software package for phylogenetic analysis. Leena Suominen acted as an expert on the greenhouse experiment. Kristina Lindström was the project leader and supervisor of the Ph.D. studies of Minna M. Jussila. Minna M. Jussila was the corresponding author of the article.

Paper IV

The experiments were planned together. Anna Kaksonen carried out the experiments, analyzed the results and wrote the first draft of the manuscript. Minna M. Jussila and Anna Kaksonen interpreted the results and wrote the final paper together. Leena Suominen acted as an expert on the field experiment. Kristina Lindström was the project leader and supervisor of the Ph.D. studies of Minna M. Jussila. Minna M. Jussila was the corresponding author of the article.

Paper V

Minna M. Jussila planned and conducted the experiments, analyzed and interpreted the results, and wrote the paper. Zhao Ji performed the tests for transconjugants, 16S rRNA gene PCR-RFLP and AEDRA analysis in supervision of Minna M. Jussila. Leena Suominen acted as an expert on the greenhouse and field experiments. Kristina Lindström was the project leader and supervisor of the Ph.D. studies of Minna M. Jussila. Minna M. Jussila was the corresponding author of the article.

Abbreviations

AEDRA	amplified enzyme-coding DNA restriction analysis
alkB	alkane monooxygenase
ATCC	American Type Culture Collection
BOX	primers corresponding to a subunit A of the BOX element
bphC	dihydroxybiphenyl dioxygenase
BTEX	benzene, toluene, ethylbenzene, xylenes
CFU	colony forming units
CLPP	community level physiological profiling
CTAB	cetyltrimethyl ammonium bromide
C23O	catechol 2,3-dioxygenase
DBT	dibenzothiophene
DGGE	denaturant gradient gel electrophoresis
DNA	deoxyribonucleic acid
DW	dry weight
EDO	extradiol aromatic ring-cleavage dioxygenase
EDTA	ethylene diaminetetraacetic acid
ERIC	enterobacterial repetitive intergenic consensus
EtBr	ethidium bromide
FISH	fluorescence *in situ* hybridization
GC-DGGE	GC (guanine and cytosine) fractionation combined with DGGE
GC-MS	gas chromatography – mass spectrometry
GTC	guanidium thiocyanate
$(GTG)_5$	a repetitive trinucleotide (guanine – thymine – guanine)
HAMBI (H)	The Microbial Culture Collection at the Division of Microbiology, Department of Applied Chemistry and Microbiology, Viikki Biocenter, University of Helsinki
HGT	horizontal gene transfer
mRNA	messenger RNA
NAH	naphthalene catabolic plasmid
nahAc	naphthalene dioxygenase
ndoB	naphthalene dioxygenase
OCT	octane catabolic plasmid
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyl
PCP	pentachlorophenol
PCR	polymerase chain reaction
PHC	petroleum hydrocarbons
PHE	phenol catabolic plasmid
Q-PCR	quantitative PCR
rDNA	ribosomal RNA gene
REP	repetitive extragenic palindromic
rep-PCR	repetitive sequence-based PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	PCR with reverse transcription
SDS	natrium (sodium) dodecyl sulphate
ssDNA	single strand DNA
THC	total hydrocarbons
TOL	toluene catabolic plasmid
UV	ultraviolet light
WHC	water holding capacity
xylE	a gene on TOL plasmid coding for C23O
16S rRNA	bacterial small subunit of rRNA

Tiivistelmä (Abstract in Finnish)

Kasvien juuristossa elävien mikrobien hyväksikäyttöä saastuneen ympäristön puhdistamisessa kutsutaan ritsoremediaatioksi. Tämän työn ajatuksena oli se, että typpeä sitovan palkokasvin, vuohenherneen (*Galega orientalis*) juuriston bakteerit voisivat osallistua 1-renkaisten aromaattisten hiilivetyjen, kuten bentseenin, tolueenin ja ksyleenin, hajottamiseen öljyllä saastuneessa maaperässä. Kemiallisen (esim. myrkkypitoisuus) ja fysikaalisen (esim. maan rakenne) tiedon lisäksi biologisten tekijöiden tuntemus (mm. bakteerit ja niiden hajotusgeenit) on oleellista kehitettäessä ritsoremediaatiosta hallittu ja tehokas biopuhdistusmenetelmä. Siksi myös luotettavien biologisten seurantamenetelmien kehittämisen tarve on ilmeinen. Tämän väitöskirjatyön tarkoituksena oli 1) arvioida vuohenherneen ja sen symbioottisen typensitojabakteerin (ritsobi, *Rhizobium galegae*) soveltuvuus öljyllä saastuneen maaperän puhdistamiseen, 2) kehittää molekyylitason menetelmiä biomonitorointia varten ja 3) soveltaa näitä menetelmiä tutkittaessa mikrobiologisia ilmiöitä ritsoremediaation aikana.

Koeputkissa vuohenherne ja sen ritsobit säilyivät elinkykyisinä jopa 3000 mg/l *m*-toluaattia sisältävässä kasvuliuoksessa. Kasvien kasvu ja nystyröinti estyivät 500 mg/l *m*-toluaattipitoisuudessa, mutta ne palautuivat, kun kasvit siirrettiin puhtaaseen alustaan. Öljyllä tai *m*-toluaatilla (2000 mg/l) saastutetussa kasvihuonekokeen maassa vuohenherne kasvoi, nystyröi ja sitoi typpeä hyvin. Sen lisäksi sen juuristo kehittyi vahvaksi. *R. galegaen* korkea aromaattisten aineiden sietokyky ja vuohenherneen elinkykyisyys öljymaissa osoitti tämän palkokasvi-bakteeri -yhdistelmän olevan lupaava systeemi öljyllä saastuneen maaperän ritsoremediaatiota kehitettäessä.

Molekulaarisia biomonitorointimenetelmiä suunniteltiin ja/tai kehitettiin bakteereille ja niiden hajotusgeeneille. Genomisen sormenjälkimenetelmän ($(GTG)_5$-PCR), 16S rRNA -geenien taksonomisen ribotyyppauksen ja 16S rRNA -geenien osittaissekvenoinnin yhdistelmä valittiin viljeltävien ja heterogeenisten juuristobakteereiden molekulaarista ryhmittelyä varten. TOL-plasmidien havaitsemiseksi suunniteltiin *xylE*-geenille spesifiset PCR-alukkeet. Sekä TOL-plasmideja (*xylE*-alukkeet) että laajemminkin erilaisia aromaatteja hajottavia entsyymejä koodaavia plasmideja (C23O-alukkeet) profiloitiin ns. monistetun, entsyymiä koodaavan DNA:n pilkonta-analyysillä (AEDRA) käyttäen *Alu*I-restriktioentsyymiä. C23O-*xylE*-PCR kehitettiin TOL-plasmidien havaitsemiseksi herkästi suoraan maasta.

Juuristobakteereita eristettiin sekä kasvihuone- että kenttäkoenäytteistä. *M*-toluaattia sietävät juuristobakteerit olivat geneettisesti hyvin erilaisia edustaen viittä bakteerien päälinjaa. Gram-positiiviset *Rhodococcus*, *Bacillus* ja *Arthrobacter* sekä gram-negatiivinen *Pseudomonas* olivat runsaimmat bakteerisuvut. *Pseudomonas putida* PaW85/pWW0 -ymppiä ei havaittu juuristonäytteissä. Vaikka tällä lisätylle bakteerille ei näyttänyt löytyvän ekologista lokeroa, sen siirtymään kykenevästä hajotusplasmidista on saattanut olla hyötyä muille bakteerilajeille ja siten myös puhdistusprosessille.

Vain 10-20% eristetyistä, *m*-toluaattia sietävistä bakteerikannoista kykeni myös hajottamaan *m*-toluaattia. TOL-plasmidit olivat näiden bakteerien hajotusplasmidien päätyyppi. Kyky hajottaa *m*-toluaattia käyttämällä TOL-plasmidin koodaamia entsyymejä havaittiin vain *Pseudomonas*-suvun lajeissa. Nämä olivat myös parhaimmat *m*-toluaatin hajottajat. Kantaspesifisiä eroja löydettiin *P. migulae* - ja *P. oryzihabitans* -lajien hajo-

tuskyvyssä: eräillä näistä kannoista oli TOL-plasmidi – mikä oli uusi löytö tässä työssä – osoittaen mahdollista horisontaalista plasmidin siirtymistä vuohenherneen juuristossa. Yhdellä *P. oryzihabitans* -kannalla oli pWW0-plasmidi, joka oli todennäköisesti konjugoitunut *Pseudomonas*-ympistä. Joillakin *P. migulae* - ja *P. oryzihabitans* -kannoilla näytti olevan sekä pWW0- että pDK1-tyyppinen TOL-plasmidi. Toisaalta niillä on saattanut olla TOL-plasmidi, jossa on sekä pWW0- että pDK1-tyyppinen *xylE*-geeni. Gram-negatiivisten bakteerien *m*-toluaatin hajotuskyky ei rajoittunut vain TOL-reittiin. Myös eräät gram-positiiviset *Rhodococcus erythropolis* - ja *Arthrobacter aurescens* -kannat kykenivät hajottamaan *m*-toluaattia ilman TOL-plasmidia.

Öljyllä saastuneen maan kenttäkokeissa tuli esiin kolme vuohenherneen juuristovaikutusta: 1) *G. orientalis* ja *Pseudomonas*-ymppi lisäsivät juuristobakteerien määrää. Vuohenherne erityisesti yhdessä *Pseudomonaksen* kanssa lisäsi *m*-toluaattia käyttävien ja katekolipositiivisten bakteerien lukumäärää osoittaen kasvillisten maiden suurempaa hajotuspotentiaalia verrattuna kasvittomiin maihin. 2) Myös bakteeridiversiteetti ribotyyppien määrällä mitattuna kasvoi vuohenherneen juuristossa sekä *Pseudomonaksen* kanssa että ilman sitä. Kuitenkaan *m*-toluaattia hajottavien bakteerien lajimäärä ei merkittävästi lisääntynyt. Myös yhteisötasolla, käyttämällä 16S rRNA -geeni-PCR-DGGE -menetelmää myös ei-viljeltävissä olevien bakteerien havainnoimiseksi, suurin bakteerilajien monimuotoisuus havaittiin kasvillisissa maissa. Monipuoliset yhteisöt saattavatkin parhaiten taata tehokkaan ritsoremediaation tarjoamalla lukuisia geneettisiä koneistoja hajotusprosessien käyttöön. 3) Kokeen lopussa TOL-plasmideja ei kyetty enää havaitsemaan suoralla DNA-analyysillä maasta, jossa oli sekä vuohenherne että *Pseudomonas*. TOL-plasmidien havaintoraja oli siis ylittynyt osoittaen hajotusplasmidien määrän vähentyneen ja viitaten siten myös ritosremediaation onnistumiseen.

Vuohenherneen käyttö ritsoremediaatiossa on ainutlaatuista. Tässä väitöskirjatyössä uutta tietoa saatiin vuohenherneen juuristovaikutuksista monoaromaattisilla hiilivedyillä saastuneissa maissa. Kehitettyjä molekulaarisia biomonitorointimenetelmiä voidaan soveltaa moneen tarkoitukseen ympäristöbioteknologian alalla, kuten maan luontaisen biohajotuspotentiaalin kartoittamiseen, bioremediaation tehostamisen seurantaan ja puhdistusprosessin onnistumisen arviointiin. Ympäristönsuojelu luonnon omia resursseja käyttäen, ja siten kestävän kehityksen periaatteen mukaisesti toimien, on sekä taloudellisesti että esteettisen ja puhtaan ympäristön säilymisen kannalta hyödyllistä yhteiskunnalle.

Avainsanat: molekyylibiologinen seuranta, geneettinen sormenjälkimenetelmä, maabakteerit, bakteerien monimuotoisuus, TOL-plasmidi, hajotusgeenit, horisontaalinen geenien siirtyminen, ritsoremediaatio, juuristovaikutus, *Galega orientalis*, hapellinen biohajotus, öljyhiilivedyt, monoaromaatit

ABSTRACT

Rhizoremediation is the use of microbial populations present in the rhizosphere of plants for environmental cleanup. The idea of this work was that bacteria living in the rhizosphere of a nitrogen-fixing leguminous plant, goat's rue (*Galega orientalis*), could take part in the degradation of harmful monoaromatic hydrocarbons, such as benzene, toluene and xylene (BTEX), from oil-contaminated soils. In addition to chemical (e.g. pollutant concentration) and physical (e.g. soil structure) information, the knowledge of biological aspects (e.g. bacteria and their catabolic genes) is essential when developing the rhizoremediation into controlled and effective bioremediation practice. Therefore, the need for reliable biomonitoring methods is obvious. The main aims of this thesis were to evaluate the symbiotic *G. orientalis – Rhizobium galegae* system for rhizoremediation of oil-contaminated soils, to develop molecular methods for biomonitoring, and to apply these methods for studying the microbiology of rhizoremediation.

In vitro, *Galega* plants and rhizobia remained viable in *m*-toluate concentrations up to 3000 mg/l. Plant growth and nodulation were inhibited in 500 mg/l *m*-toluate, but were restored when plants were transferred to clean medium. In the greenhouse, *Galega* showed good growth, nodulation and nitrogen fixation, and developed a strong rhizosphere in soils contaminated with oil or spiked with 2000 mg/l *m*-toluate. The high aromatic tolerance of *R. galegae* and the viability of *Galega* plants in oil-polluted soils proved this legume system to be a promising method for the rhizoremediation of oil-contaminated soils.

Molecular biomonitoring methods were designed and/or developed further for bacteria and their degradation genes. A combination of genomic fingerprinting ($(GTG)_5$-PCR), taxonomic ribotyping of 16S rRNA genes and partial 16S rRNA gene sequencing were chosen for molecular grouping of culturable, heterogeneous rhizosphere bacteria. PCR primers specific for the *xylE* gene were designed for TOL plasmid detection. Amplified enzyme-coding DNA restriction analysis (AEDRA) with *Alu*I was used to profile both TOL plasmids (*xylE* primers) and, in general, aromatics-degrading plasmids (C230 primers). The sensitivity of the direct monitoring of TOL plasmids in soil was enhanced by nested C23O-*xylE*-PCR.

Rhizosphere bacteria were isolated from the greenhouse and field lysimeter experiments. High genetic diversity was observed among the 50 isolated, *m*-toluate tolerating rhizosphere bacteria in the form of five major lineages of the Bacteria domain. Gram-positive *Rhodococcus*, *Bacillus* and *Arthrobacter* and gram-negative *Pseudomonas* were the most abundant genera. The inoculum *Pseudomonas putida* PaW85/pWW0 was not found in the rhizosphere samples. Even if there were no ecological niches available for the bioaugmentation bacterium itself, its conjugative catabolic plasmid might have had some additional value for other bacterial species and thus, for rhizoremediation.

Only 10 to 20% of the isolated, *m*-toluate tolerating bacterial strains were also able to degrade *m*-toluate. TOL plasmids were a major group of catabolic plasmids among these bacteria. The ability to degrade *m*-toluate by using enzymes encoded by a TOL plasmid was detected only in species of the genus *Pseudomonas*, and the best *m*-toluate degraders were these *Pseudomonas* species. Strain-specific differences in degradation abilities were found for *P. oryzihabitans* and *P. migulae*: some of these strains harbored

a TOL plasmid – a new finding observed in this work, indicating putative horizontal plasmid transfer in the rhizosphere. One *P. oryzihabitans* strain harbored the pWW0 plasmid that had probably conjugated from the bioaugmentation *Pseudomonas*. Some *P. migulae* and *P. oryzihabitans* strains seemed to harbor both the pWW0- and the pDK1-type TOL plasmid. Alternatively, they might have harbored a TOL plasmid with both the pWW0- and the pDK1-type *xylE* gene. The breakdown of *m*-toluate by gram-negative bacteria was not restricted to the TOL pathway. Also some gram-positive *Rhodococcus erythropolis* and *Arthrobacter aurescens* strains were able to degrade *m*-toluate in the absence of a TOL plasmid.

Three aspects of the rhizosphere effect of *G. orientalis* were manifested in oil-contaminated soil in the field: 1) *G. orientalis* and *Pseudomonas* bioaugmentation increased the amount of rhizosphere bacteria. *G. orientalis* especially together with *Pseudomonas* bioaugmentation increased the numbers of *m*-toluate utilizing and catechol positive bacteria indicating an increase in degradation potential. 2) Also the bacterial diversity, when measured as the amount of ribotypes, was increased in the *Galega* rhizosphere with or without *Pseudomonas* bioaugmentation. However, the diversity of *m*-toluate utilizing bacteria did not significantly increase. At the community level, by using the 16S rRNA gene PCR-DGGE method, the highest diversity of species was also observed in vegetated soils compared with non-vegetated soils. Diversified communities may best guarantee the overall success in rhizoremediation by offering various genetic machineries for catabolic processes. 3) At the end of the experiment, no TOL plasmid could be detected by direct DNA analysis in soil treated with both *G. orientalis* and *Pseudomonas*. The detection limit for TOL plasmids was encountered indicating decreased amount of degradation plasmids and thus, the success of rhizoremediation.

The use of *G. orientalis* for rhizoremediation is unique. In this thesis new information was obtained about the rhizosphere effect of *Galega orientalis* in BTEX contaminated soils. The molecular biomonitoring methods can be applied for several purposes within environmental biotechnology, such as for evaluating the intrinsic biodegradation potential, monitoring the enhanced bioremediation, and estimating the success of bioremediation. Environmental protection by using nature's own resources and thus, acting according to the principle of sustainable development, would be both economically and environmentally beneficial for society.

Keywords: molecular biomonitoring, genetic fingerprinting, soil bacteria, bacterial diversity, TOL plasmid, catabolic genes, horizontal gene transfer, rhizoremediation, rhizosphere effect, *Galega orientalis*, aerobic biodegradation, petroleum hydrocarbons, BTEX

1 INTRODUCTION

1.1 Bioremediation: the use of living organisms for the remediation of polluted sites

Growing awareness of the harmful effects of pollution to the environment and human health has led to a marked increase in research into various strategies that might be used to clean up contaminated sites. Many conventional, engineering-based decontamination methods are expensive partially because of the cost of excavating and transporting large quantities of contaminated materials for *ex situ* treatment, such as soil washing, chemical inactivation, and incineration (Chaudhry et al., 2005; Pilon-Smits, 2005). The increasing costs and limited efficiency of these traditional **physicochemical treatments** of soil have spurred the development of alternative technologies for *in situ* applications, in particular those based on biological remediation capabilities of plants and microorganisms (Chaudhry et al., 2005; Singh & Jain, 2003). The term **BIOREMEDIATION** refers to the use of living organisms to degrade environmental pollutants (Barea et al., 2005; Kuiper et al., 2004; Dua et al., 2002).

1.1.1 Intrinsic bioremediation, natural attenuation

The simplest form of bioremediation is **natural attenuation** (NA) or **BIOATTENUATION**, during which the indigenous microbial populations degrade recalcitrants or xenobiotics based on their natural, nonengineered metabolic processes (Kuiper et al., 2004; Widada et al., 2002). According to the Environmental Protection Agency in the United States (USEPA 1999) NA or **intrinsic bioremediation** processes include a variety of physical, chemical, and biological processes that act to reduce the mass, toxicity, mobility, volume, or concentration of contaminants. These processes include aerobic and anaerobic biodegradation, dispersion, dilution, sorption, volatilization, radioactive decay, and chemical or biological stabilization, transformation, or destruction of contaminants (Rittmann, 2004). The purpose in **monitored natural attenuation** (MNA) is to use both chemical parameters (such as the concentration of xenobiotics, intermediate formation, end product formation, electron acceptor consumption, and toxicity) and biological parameters (such as the composition, size, and degrading activity of the microbial populations) to follow the natural degradation processes of microbes (Kuiper et al., 2004). Subsequently, estimations could be made about the degradation rate in combination with the spread of the contamination plume. NA can well be applied on sites with low environmental or public value, when time is not a limiting factor and where no other restoration techniques are applicable (Kuiper et al., 2004). As a limiting factor, microbes with suitable catabolic genes might not be available on the site.

1.1.2 Biostimulation

The intentional stimulation of resident xenobiotic degrading bacteria by addition of electron acceptors, water, nutrients, or electron donors, in order to speed up the bioremediation process, is referred to as **BIOSTIMULATION** (Widada et al., 2002; Madsen, 1991). In many cases, however, fertilization practice of contaminated sites using compost, nitrogen, phosphorous, and carbon has been unpredictable because it has been reported to either enhance or not the degradation of pollutants (Kuiper et al., 2004; Brodkorb & Legge, 1992; Namkoong et al., 2002; Ramadan et al., 1990; Wang et al., 1990).

1.1.3 Bioaugmentation

BIOAUGMENTATION is a method to improve degradation and enhance the transformation rate of xenobiotics by introducing either WILD-TYPE or GENETICALLY MODIFIED microbes into soil (Kuiper et al., 2004). A bioinoculant or bioinoculate is a beneficial, natural microbe introduced into soil by e.g. coating of seeds or in a peat preparation. The laboratory scale results of seeding microbes for degradation of soil pollutants have been ambiguous (Kuiper et al., 2004). Possible reasons for

the failure of bacterial inoculation could be one or more of the following (Kuiper et al., 2004; Goldstein et al, 1985). First, the concentration of the contaminants at a site can be too low to support growth of the inoculum. This also includes the problem of low bioavailibility of certain pollutants. Second, the presence of certain compounds in the environment can inhibit the growth or activity of the inoculum. Third, protozoan-grazing rates on the inoculum can be higher than the growth rates of the bacteria, resulting in a decline of the bacterial population. Fourth, the inoculum can prefer to use other carbon sources present in soil, instead of the contaminant. Fifth, inocula may fail because of the inability of the microbes to spread through the soil and reach the pollutant. Co-inoculation of a CONSORTIUM of bacteria, each with different parts of the catabolic degradation route, involved in the degradation of a certain pollutant is often found to be more efficient than the introduction of one single strains with the complete pathway (Kuiper et al., 2004; Rahman et al., 2002).

1.1.4 Phytoremediation

PHYTOREMEDIATION refers to the use of plants for bioremediation. Often it is expanded to mean the use of plants and their associated microbes for environmental cleanup (Pilon-Smits, 2005; Salt et al., 1998; Singh & Jain, 2003). Phytoremediation is easy to implement, and both the set-up and maintenance are cost-effective. This noninvasive method is also sustainable and can be visually attractive. On other hand, soil properties and climate can restrict plant growth. Different ways to phytoremediate are illustrated in Fig. 1-1. Possible fates of pollutants during phytoremediation are presented in Fig. 1-2 A. These different PHYTOTECHNOLOGIES make use of different plant properties and typically different plant species are used for each (Fig. 1-2 B). Favourable PLANT PROPERTIES

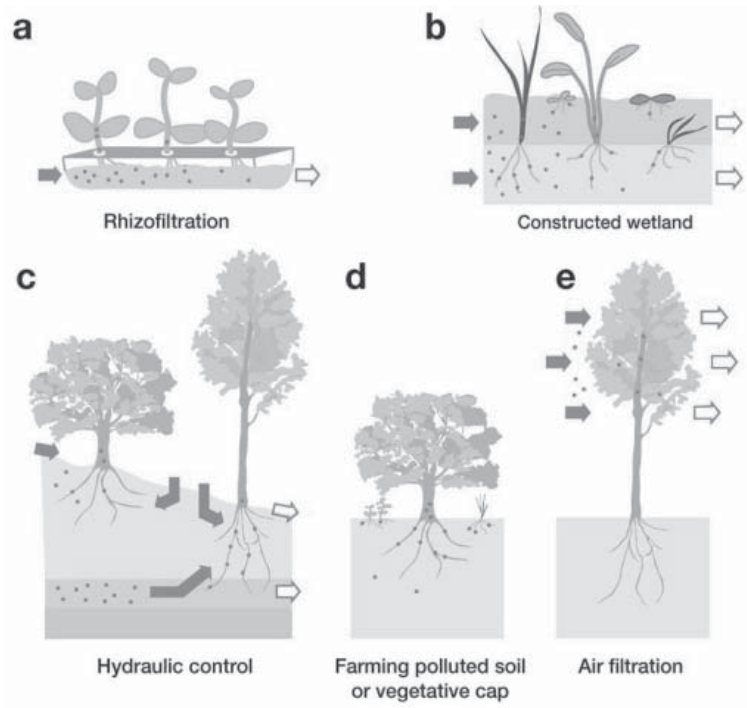
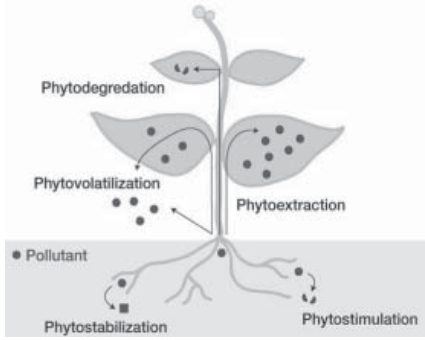


Fig. 1-1. Phytoremediation technologies used for remediating polluted water, soil, or air (Pilon-Smits, 2005). The circles represent the pollutant. Reprinted, with permission, from the Annual Review of Plant Biology, Volume 56 (c) 2005 by Annual Reviews www.annualreviews.org. Rhizofiltration uses plants, such as sunflower (*Helianthus annuus*) or water hyacinth (*Eichhornia crassipes*), in hydroponic setup for filtering mainly inorganics, such as metals and radionuclides, from polluted water.

A



B

Plant shoots

Phytodegradation

* the breakdown of pollutants by plant enzymes, usually inside tissues (but also in soil by laccases, dehalogenases, nitroreductases, nitrilases, and peroxidases)
* mobile organics: herbicides, TNT, MTBE, TCE
* poplar (*Populus* sp.)

Phytovolatilization

* the release of pollutants by plants in volatile form
* VOCs such as TCE and MTBE, and volatile inorganics such as Se and Hg
* poplar (*Populus* sp.), *Brassica* (Se)

Phytoextraction

* the use of plants to clean up pollutants via accumulation (sequestration) in harvestable tissues
  phytomining, recycling of the accumulated element
* metals and other toxic inorganics (Se, As, radionuclides)
* Indian mustard (*Brassica juncea*); Ni hyperaccumulator *Alyssum bertolonii*, As hyperaccumulator fern (*Pteris vittata*)

Rhizosphere

Phytostabilization

* the use of plants to stabilize pollutants in soil by either preventing erosion, leaching, or runoff (hydraulic barrier, buffer strips), or by converting pollutants to less bioavailable forms (e.g. via precipitation in the rhizosphere)
* organics and inorganics
* poplar (*Populus* sp.), grasses

Rhizoremediation

a. Phytostimulation

* the stimulation of microbial degradation processes in the rhizosphere by plant enzymes, secondary plant compounds (e.g. terpenes, saponins), or other compounds in root exudate
* hydrophobic organics such as PCBs and PAHs, and other petroleum hydrocarbons
* mulberry trees (*Morus* sp.) with phenolic compounds and flavonones

b. Rhizodegradation

* the degradation of pollutants in the rhizosphere due to microbial activity
* hydrophobic organics such as PCBs and PAHs, and other petroleum hydrocarbons
* grasses such as fescue (*Festuca* sp.) and ryegrass (*Lolium* sp.)

Fig. 1-2 A. Possible fates of pollutants during phytoremediation (Pilon-Smits, 2005). Reprinted, with permission, from the Annual Review of Plant Biology, Volume 56 (c) 2005 by Annual Reviews www.annualreviews.org. **B.** The principle of each phytotechnology with examples of typical pollutants and plant species used. Data collected from Pilon-Smits (2005), Salt et al. (1998) and Chaudhry et al. (2005). MTBE, methyl tertiary butyl ether; PAH, polyaromatic hydrocarbon; PCB, polychlorinated biphenyl; TCE, trichloroethylene; TNT, trinitrotoluene; VOC, volatile organic compound.

for phytoremediation in general are fast growth rate, high biomass production, competitiveness, hardiness, and tolerance of pollution (Pilon-Smits, 2005). In addition, high levels of plant uptake, translocation, and accumulation in harvestable tissues are important properties for the phytoextraction of inorganics. Favourable plant properties for rhizo- and phytodegradation are large, dense, and deep root systems and high levels of degrading enzymes, respectively. A large root surface area also favours phytostimulation (e.g. plant root enzymes), and promotes microbial growth; furthermore, production of specific exudate compounds may further promote rhizodegradation via specific plant-microbe interactions (Pilon-Smits, 2005). Soils contaminated with organic chemicals often also contain toxic heavy metals. Under such conditions, mycorrhizas, symbiotic root-fungal associations, may play a major role in phytoremediation (Chaudhry et al., 2005). Some ectomycorrhizal species are particularly efficient in degrading recalcitrant organic compounds, such as BTEX, and their capacity is retained even under symbiotic conditions in soil (Sarand et al., 1999).

Phytoremediation can be applied to both organic and inorganic pollutants present as solid, liquid, or gaseous substrates (Pilon-Smits, 2005; Salt et al., 1998). Organic pollutants in the environment are mostly man made and xenobiotic. Many of them are toxic, some carcinogenic. Organic pollutants are released into the environment via spills (fuel, solvents), military activities (explosives, chemical weapons), agriculture (pesticides, herbicides), industry (chemical, petrochemical), wood treatment, etc. (Pilon-Smits, 2005). Depending on their properties, organics may be degraded in the root zone of plants or taken up, followed by degradation, sequestration, or volatilization (Pilon-Smits, 2005). Organic pollutants that have been successfully phytoremediated include organic solvents such as TCE (trichloroethylene), herbicides such as atrazine, explosives such as TNT (trinitrotoluene), petroleum hydrocarbons such as oil, gasoline, BTEX (monoaromatic hydrocarbons), and PAHs (polyaromatic hydrocarbons), the fuel additive MTBE (methyl tertiary butyl ether), and PCBs (polychlorinated biphenyls) (Pilon-Smits, 2005). Inorganic pollutants occur as natural elements, and human activities such as mining and traffic promote their release into the environment, leading to toxicity (Pilon-Smits, 2005). Inorganics cannot be degraded, but they can be phytoremediated via stabilization or sequestration in harvestable plant tissues (Pilon-Smits, 2005). Inorganic pollutants that can be phytoremediated include plant macronutrients such as nitrate and phosphate, plant trace elements such as Cr and Zn, nonessential elements (heavy metals) such as Cd and Hg, and radioactive isotopes such as ^{238}U and ^{90}Sr (Pilon-Smits, 2005). The main application for phytoremediation has so far been to remove toxic heavy metals from soil (Chaudhry et al., 2005; Salt et al., 1998). However, there is a growing interest in broadening applications of the technology to remove/degrade organic pollutants in the environment.

1.1.5 Rhizoremediation: the use of beneficial plant-microbe interactions for environmental cleanup

Without the microbial contribution, phytoremediation alone may not be a viable technology for many hydrophobic organic pollutants (Chaudhry et al., 2005). The use of rhizomicrobial populations present in the rhizosphere of plants for bioremediation is referred to as **rhizoremediation** (Anderson et al., 1993; Kuiper et al., 2004). The term consists of both phytostimulation and rhizodegradation describing, thus, the importance of both the plant and the microbes in this beneficial interaction. Plant-microbial interactions in the rhizosphere offer very useful means for remediating environments contaminated with recalcitrant organic compounds (Chaudhry et al., 2005). The success of a plant species as the spot of rhizoremediation might depend on 1) highly branched root system to harbor large numbers of bacteria, 2) primary and secondary metabolism, and 3) establishment, survival, and ecological interactions with other organisms (Kuiper et al., 2004; Salt et al., 1998). Plant roots can act as a substitute for the tilling of soil to incorporate additives (nutrients) and to improve aeration (Kuiper et al., 2004; Aprill & Sims, 1990).

Various grass varieties and leguminous plants have shown to be suitable for rhizoremediation (Kuiper et al., 2001, 2004). Species and hybrids between species belonging to the genera *Populus*

sp. (poplar) and *Salix* sp. (willow) have been used successfully for rhizoremediation of PHC-contaminated soils probably due to introduction of oxygen into deeper soil layers through specialized root vessels, aerenchyma (Zalesny et al., 2005). Thoma et al. (2003) would prefer annual grass species with high root turnover for PHC-contaminated soils. Many characteristics of goat's rue (*Galega orientalis*) would support its choice for rhizoremediation. The root system of *G. orientalis* is well developed and the roots will penetrate to at least one meter below ground (Varis, 1986). Since this legume plant overwinters with the aid of underground stolons, it is not susceptible to attack by pathogenic fungi in the space between the ground and the snow, and will survive for several years in a northern temperate climate. In addition, the cultivation practice for the plant has been optimized and it is especially used in ecological cropping systems. *G. orientalis* forms a very specific symbiosis with *Rhizobium galegae* enabling the plant to grow in nitrogen-poor soil (Lindström, 1989).

The mucigel secreted by root cells, lost root cap cells, the starvation of root cells, or the decay of complete roots provides nutrients in the rhizosphere (Kuiper et al., 2004; Lugtenberg & de Weger, 1992; Lynch & Whipps, 1990). In addition, plants release a variety of photosynthesis-derived organic compounds (Pilon-Smits, 2005; Salt et al., 1998). These ROOT EXUDATES contain water soluble, insoluble, and volatile compounds including sugars, alcohols, amino acids, proteins, organic acids, nucleotides, flavonones, phenolic compounds and certain enzymes (Chaudhry et al., 2005; Pilon-Smits, 2005; Salt et al., 1998; Anderson et al., 1993). The rate of exudation changes with the age of a plant, the availability of mineral nutrients and the presence of contaminants (Chaudhry et al., 2005). The nature and the quantity of root exudates, and the timing of exudation are crucial for a rhizoremediation process. The root exudates mediate acquisition of minerals by plants and stimulate microbial growth and activities in the rhizosphere in addition to changing some physicochemical conditions. Plants might respond to chemical stress in the soil by changing the composition of root exudates controlling, in turn, the metabolic activities of rhizosphere microorganisms (Chaudhry et al., 2005). Some organic compounds in root exudates may serve as carbon and nitrogen sources for the growth and long-term survival of microorganisms that are capable of degrading organic pollutants (Pilon-Smits, 2005; Salt et al., 1998; Anderson et al., 1993). For instance, plant phenolics such as catechin and coumarin may serve as co-metabolites for PCB-degrading bacteria (Pilon-Smits, 2005; Chaudhry et al., 2005; Salt et al., 1998). **Co-metabolism** is defined as the oxidation of nongrowth substrates during the growth of an organism on another carbon or energy source (Kuiper et al., 2004). Some co-metabolized recalcitrant pollutants such as the pesticide lindane (organochlorine) are only transformed and not effectively mineralized by microorganisms (Paul et al., 2005).

Microbes living in the rhizosphere, **RHIZOMICROBIA**, in turn, can promote plant health by stimulating root growth (regulators), enhancing water and mineral uptake, and inhibiting growth of pathogenic or other, non-pathogenic soil microbes (Pilon-Smits, 2005; Kuiper et al., 2004). The microbial transformations of organic compounds are usually not driven by energy needs, but a necessity to reduce toxicity due to which microbes may have to suffer an energy deficit (Chaudhry et al., 2005). Thus, the processes may be enhanced or driven by the abundant energy that is provided by root exudates. Such stimulation of soil microbial communities by root exudates also benefits plants through increased availability of soil-bound nutrients and degradation of phytotoxic soil contaminants (Chaudhry et al., 2005). This might allow the spread of roots into deeper soil layers. Rhizomicrobia may also accelerate remediation processes by volatilizing organics such as PAHs or by increasing the humification of organic pollutants (Salt et al, 1998). In particular, the release of oxidoreductase enzymes (e.g. peroxidase) by microbes, as well as by plant roots, can catalyze the polymerization of contaminants onto the soil humic fraction and root surfaces.

In contrast to the limited studies of rhizoremediation (Kuiper et al., 2004), beneficial plant growth promoting, root-colonizing rhizobacterial strains (PGPR) have been extensively described for processes such as biocontrol of plant pathogens, and nutrient cycling by nonsymbiotic N_2-fixing bacteria and phosphate solubilizing bacteria (PSB), i.e. biofertilization (reviewed by Barea et al., 2005). The success of beneficial processes is based on the **RHIZOSPHERE COMPETENCE** of

the microbes, which is reflected by the ability of the microbes to survive in the rhizosphere, compete for the exudate nutrients, sustain in sufficient numbers, and efficiently colonize the growing root system (Kuiper et al., 2004; Lugtenberg & Dekkers, 1999). Fairly recently, it was shown that chemotaxis by *P. fluorescens* WCS365 toward some organic acids and amino acids (but not to sugars) present in tomato root exudate plays an important role during root colonization (Kuiper et al., 2004; de Weert et al., 2002). Another example of the importance of the efficient use of exudates was shown for *P. putida* PCL1444. The selection of this strain for survival and proliferation on grass roots coincided with very efficient use of the main organic acids and sugars from the grass root exudate and with a high expression level of its catabolic genes for naphthalene degradation during the use of these substrates (Kuiper et al., 2002, 2004).

Usually, several bacterial populations degrade pollutants more efficiently than a single species/strain due to the presence of partners, which use the various intermediates of the degradation pathway more efficiently (JOINT METABOLISM) (Kuiper et al., 2004; Pelz et al., 1999). During rhizoremediation, the degradation of a pollutant, in many cases, is the result of the action of a consortium of bacteria (Kuiper et al., 2004). The colonization of different niches of plant roots by different strains has also been recognized (Kuiper et al., 2001, 2004; Dekkers et al., 2000). Interestingly, the close proximity of the different strains and the formation of mixed microcolonies were observed only in the presence of the pollutant, naphthalene illustrating the formation of communities where different activities fulfil each other (Kuiper et al., 2001, 2004). However, very few studies report the directed introduction of a microbial strain or consortium for xenobiotic degradation activities (**bioaugmented rhizoremediation**), which is able to efficiently colonize the root (Kuiper et al., 2001; 2004; Ronchel & Ramos, 2001; Sriprang et al., 2002).

1.1.5.1 Rhizosphere effect

The **rhizosphere** is the zone of soil around the root in which microbes are influenced by the root system forming a dynamic root-soil interface (Kuiper et al., 2004; Pilon-Smits, 2005; Barea et al., 2005). There are three separate, but interacting, components recognized in the rhizosphere: 1) rhizosphere (soil), the zone of soil influenced by roots through the release of substrates that affect microbial activity, 2) rhizoplane, the root surface, including the strongly adhering soil particles, and 3) root tissue that some endophytic microorganisms (endophytes) are able to colonize (Barea et al., 2005). The differing physical, chemical, and biological properties of the root-associated soil, compared with those of the bulk soil, are responsible for changes in microbial diversity and for increased numbers and metabolic activities of microorganisms in the rhizosphere micro-environment, the phenomenon called the RHIZOSPHERE EFFECT (Barea et al., 2005; Kuiper et al., 2004; Pilon-Smits, 2005; Salt et al., 1998). Densities of rhizospheric bacteria can be as much as two to four orders of magnitude greater than populations in the surrounding bulk soils and display a greater range of metabolic capabilities, including the ability to degrade a number of recalcitrant xenobiotics (Pilon-Smits, 2005; Salt et al., 1998). It is not surprising, therefore, to find ACCELERATED rates of BIODEGRADATION of organic pollutants in vegetated soils compared with nonvegetated soils.

1.1.5.2 Pollutant bioavailability

The bioavailability of organic compounds is the most important factor that determines the overall SUCCESS OF A BIOREMEDIATION process (Chaudhry et al., 2005; Salt et al., 1998). **POLLUTANT BIOAVAILABILITY** depends on the chemical properties of the pollutant (hydrophobicity, volatility), (heterogeneous) soil properties (particle size, water and organic content, cation exchange capacity, pH), environmental conditions (oxygen, temperature, moisture), and biological activity (plants, microbes) (Pilon-Smits, 2005; Salt et al., 1998). Rentz et al. (2003) suggested that overcoming oxygen limitation to plants should be considered in phytoremediation projects when soil contamination exerts a high biochemical oxygen demand, such as in former oil refinery sites. Plants with aerenchyma such as reed (*Phragmites australis*) can release oxygen into the rhizosphere and are used for rhizoremediation (Muratova et al., 2003).

Various PLANT AND/OR MICROBIAL ACTIVITIES effect on pollutant bioavailability. Surfactants are amphipathic molecules with both a hydrophobic and a hydrophilic part (Kuiper et al., 2004; Pilon-Smits, 2005; Salt et al., 1998). Many microbes can produce such surface-active agents, referred to as BIOSURFACTANTS, to enhance the water solubility and bacterial degradation of organic contaminants (Kuiper et al., 2004; Salt et al., 1998). Some *Pseudomonas aeruginosa* strains produce rhamnolipids (Kuiper et al., 2004; Noordman & Jansen, 2002; Providenti et al., 1995), some *Bacillus* spp. strains surfactins (Kuiper et al., 2004; Fuma et al., 1993; Yakimov et al., 1995) and some *P. fluorescens* strains cyclic lipopeptides (Nielsen & Sørensen, 2003).

PLANT ROOT EXUDATES OR LYSATES may also contain lipophilic compounds that increase pollutant water solubility (PLANT SURFACTANTS) or promote biosurfactant-producing microbial populations (Read et al., 2003; Pilon-Smits, 2005). Furthermore, PLANT- AND MICROBE-DERIVED ENZYMES can affect the solubility and thus the bioavailability of organic pollutants via modification of side groups. Plants and microbes seem to have a limited ability to degrade certain organic compounds, in particular those containing chlorines and/or aromatic ring structures (Chaudhry et al., 2005). SECONDARY PLANT COMPOUNDS, such as phenolics, released from roots may specifically induce microbial genes involved in degradation of organics (PCB, PAH), or act as a co-metabolite to facilitate microbial degradation (Pilon-Smits, 2005; Fletcher & Hegde, 1995; Leigh et al., 2002). In other words, microorganisms capable of using phenolic compounds as a carbon source often have enzymes that can co-metabolize pollutants with similar structures (Chaudhry et al., 2005).

Some natural organic acids such as citrate and malate, secondary plant compounds such as phenolics, and siderophores exuded by plants (PHYTOSIDEROPHORES) and bacteria can act as CHELATORS of metal ions to release metal cations from soil particles (Pilon-Smits, 2005; Salt et al., 1998; Chaudhry et al., 2005). Some plant roots (*Salix* sp., *Populus* sp., some aquatic plants) release oxygen that may lead to the oxidation of metals to insoluble forms (e.g. FeO_3) that precipitate on the root surface (a form of PHYTOSTABILIZATION) (Pilon-Smits, 2005; Chaudhry et al., 2005).

Chemotaxis has been shown to promote bioavailability in bacteria isolated from a polluted rhizosphere that degrade PAHs (Paul et al., 2005; Ortega-Calvo et al., 2003). CHEMOTAXIS is the movement of microorganisms under the influence of a chemical gradient that helps them to find optimum conditions for growth and survival (Paul et al., 2005). In the past few years, several microbes have been reported to be chemotactic towards different environmental pollutants, for instance toluene acting as chemoattractant to *Pseudomonas putida* F1 (Paul et al., 2005; Parales et al., 2000). Chemotactic bacteria might be more competent for bioremediation than their non-chemotactic counterparts (Paul et al., 2005).

1.1.5.3 Potential ways of enhancing the efficiency of rhizo- and phytoremediation

The most important factor to enhance bioremediation is the pollutant BIOAVAILABILITY as mentioned before (ch. 1.1.5.2). ARTIFICALLY, the pollutant bioavailability could be improved by adding soil amendments in the form of surfactants, such as Triton X-100 and SDS (Kuiper et al., 2004; Pilon-Smits, 2005; Salt et al., 1998). EDTA can be used for chelate-assisted phytoextraction of metals (Pilon-Smits, 2005; Salt et al., 1998). In addition, the deposition of specific phytochemicals and synthetic plant root exudates in soil offers the potential for developing '**rhizosphere breeding**' as a method to stimulate the growth and activity of beneficial soil microbes. In the following, a few other ways for enhancement are presented.

In soils polluted with organic chemicals, a COMBINED STRESS might enhance the degradation (Chaudhry et al., 2005). For example, moderate nutritional or water stress is known to induce root proliferation and enhance root hair density as a means of acquiring additional nutrients and water. This may enhance the interface area between soil and plants, and consequently the potential for enhancing root-mediated effects on pollutant degradation. In addition, P deficiency may be exploited to increase co-metabolism of recalcitrant organic pollutants that is driven by root exudates (Chaudhry et al., 2005). An induction of stress in plants, with known effects on root

functioning, may also offer a tool for studying the factors that stimulate biodegradation of organic pollutants (Chaudhry et al., 2005).

In the field of ROOT TECHNOLOGY, certain strains of the naturally-occurring soil bacterium *Agrobacterium tumefaciens* has been used to induce root proliferation ('**plant breeding**') in order to increase the length and mass of plant roots and thus the degradation (Chaudhry et al., 2005; Stomp et al., 1994). Thus, 'plant breeding' may also help 'rhizosphere breeding'.

An INCREASE IN THE ACTIVITIES OF INDIGENOUS MICROBIAL SPECIES in a rhizosphere is likely to enhance the degradation of soil contaminants due to co-metabolic processes of diauxic growth (Chaudhry et al., 2005). Thus, provision of appropriate nutrients and growth conditions may have a significant impact on microbial numbers in the rhizosphere and consequently the remediation of contaminated soils (BIOSTIMULATION). The rates of degradation of 3- and 4-ring PAHs were the highest when artificial root exudates with N were added to the soil (Joner et al., 2002). Further addition of P led to an increase in the rate of degradation of 5-ring PAHs, as well as an increase in the number of PAH degrading bacteria in soil. Application of fertilisers and increased moisture content of soil, or application of organic manure, has also been reported to increase the total number of microorganisms in plant rhizospheres (Chaudhry et al., 2005). However, microbial composition has been reported to be different in soils fertilised with organic or inorganic fertilisers.

Selective introduction of microorganisms with specific or broad-ranging biodegrading properties into a plant rhizosphere (BIOAUGMENTED RHIZOREMEDIATION) may accelerate remediation of soils polluted with organic chemicals (Chaudhry et al., 2005). The best-suited microbes for bioremediation can be expected to be those isolated from sites contaminated with a particular target compound. The bacterial species that are known to be the most useful in bioremediation belong to the genera *Flavobacterium*, *Arthrobacter*, *Azotobacter*, *Pseudomonas* and *Burkholderia* (Chaudhry et al., 2005). Indeed, studies have shown that a number of *Burkholderia* species exist naturally in the rhizosphere of several crop plants (reviewed by Tabacchioni et al., 2002). The inoculation of rhizospheres with (a) particular pollutant-degrading microorganism(s) has been shown to lead to an enhancement of biological remediation (Chaudhry et al., 2005). For example, after the introduction of a microbial consortium derived from a contaminated site, the crested wheatgrass (*Agropyron desertorum*) showed up to 10-fold greater tolerance to PCP, compared to sterile plants (Chaudhry et al., 2005; Miller et al., 1998).

GENETIC ALTERATIONS OF PLANTS AND TRANSGENIC PLANTS for improved phytoremediation have already been developed and are spreading for field studies (Pilon-Smits, 2005; Chaudhry et al., 2005). To selectively bind specific organic contaminants in the environment, the expression of single chain variable fragment (scFv) of immunoglobulins that contains the binding site of an antibody towards a specific analyte (plantibodies) or receptors in plants or rhizosphere microorganisms could be engineered in the future (Chaudhry et al., 2005).

The transfer of the *tfdA* gene (encoding 2,4-dichlorophenoxyacetic acid/2-oxoglutarate dioxygenase) in a plasmid to phenol-degrading bacteria (*Ralstonia eutropha* and *Pseudomonas* sp.) has been reported to significantly increase their ability to degrade phenoxyacetic acid in sterile and non-sterile soil microcosms of a sandy loam soil (Lipthay et al., 2001). Recently, an endophytic microorganism *Burkholderia cepacia* of yellow lupine was genetically manipulated to enhance toluene degradation (Pilon-Smits, 2005; Barac et al., 2004). *B. cepacia* was transformed with a plasmid from a related strain containing genes that mediate toluene degradation. After infection of lupine with the modified strain, the resulting plants were more tolerant to toluene and volatilized less of it through the leaves. This was the first example of GENETIC MODIFICATION of an endophyte for rhizoremediation.

In all, to increase the efficiency of phyto- and rhizoremediation, there is still a need for better KNOWLEDGE OF THE BIOLOGICAL PROCESSES involved that affect pollutant bioavailability, plant-microbe interactions and other rhizosphere processes, plant uptake, translocation mechanisms, tolerance mechanisms (compartmentation, degradation), chelation and volatilization (Pilon-Smits, 2005; Chaudhry et al., 2005). In coming years, novel genes important for bioremediation will be searched from both plants and bacteria (Pilon-Smits, 2005). Further research is also needed to

investigate different feedback mechanisms that select and regulate the plant-microbe interactions and microbial activity in the rhizosphere (Chaudhry et al., 2005).

1.1.6 Challenges for future bioremediation practices

Continued bioremediation research should benefit from a multidisciplinary approach, involving teams with expertise at different levels, to study the remediation of pollutants from the molecule to the ecosystem (Pilon-Smits, 2005). In addition, multidisciplinary treatment methods combining both engineering-based and bioremediation techniques might be the most effective solution (Kuiper et al., 2004; Pilon-Smits, 2005). In order to DESIGN the most EFFECTIVE METHODOLOGY of soil treatment, and to best decide which technique(s) will be applied in each case, an elaborate STUDY OF THE SITE should be made (Kuiper et al., 2004). Important parameters for bioremediation are 1) the nature of the pollutants, 2) the soil structure and hydrogeology (movement of pollutants through soil and ground water), and 3) the nutritional state and microbial composition of the site (Kuiper et al., 2004; Dua et al., 2002; Blackburn and Hafker, 1993; Long 1993). To become a generally accepted technique, the effectiveness and predictability of bioremediation should be demonstrated (Kuiper et al., 2004). Therefore, the NEED FOR RELIABLE MONITORING METHODS AND EVALUATION CRITERIA is obvious.

1.2. Aerobic biodegradation of petroleum hydrocarbons

1.2.1 Petroleum hydrocarbons

PETROLEUM, i.e. **crude oil**, is an extremely complex mixture of hydrocarbons (Atlas, 1981). Crude oil can be distilled into various OIL REFINERY PRODUCTS such as gasoline (benzine), diesel oil, petrol, kerosene, paraffin, bitumen and asphalt still consisting of a mixture of hydrocarbons. The petroleum mixture can be FRACTIONATED into a saturated or **aliphatic** fraction, an **aromatic** fraction, and an asphaltic or **polar** fraction (Atlas, 1981). Hydrocarbons within the saturated fraction include *n*-alkanes, branched alkanes, and cycloalkanes (naphthenes). The *n*-alkanes are generally considered the most readily degraded components in a petroleum mixture. The aerobic bacterial degradation of aromatic compounds involves (as chromosomally encoded) the formation of a diol followed by cleavage and formation of a diacid such as *cis*,*cis*-muconic acid. The metabolic pathways for the degradation of asphaltic components of petroleum are least well understood.

1.2.1.1 Aromatic hydrocarbons

Aromatic hydrocarbons, i.e. **arenes** or **AROMATICS**, are hydrocarbons that consist of a ring structure in plane (Fig. 1-3). Many aromatics are carcinogenic. The biodegradation of aromatic compounds can be considered, on the one hand as part of the normal process of the carbon cycle, and as the removal of man-made pollutants from the environment, on the other (Smith, 1990). Because aromatic hydrocarbons are also naturally occuring organic compounds, it is not surprising that diverse microorganisms have evolved the ability to utilize these compounds (Atlas, 1981).

MONOAROMATICS contain one aromatic ring and they are highly soluble to organic solvents (Assinder & Williams, 1990). The parent member of the aromatic hydrocarbons is **benzene** (Smith, 1990). During aerobic biodegradation, benzene is oxidized to catechol (dihydroxylated benzene), which is further catabolized by either catechol 1,2-dioxygenase (the *ortho*- or intradiol-cleavage) and subsequently via the β–ketoadipate pathway or catechol 2,3-dioxygenase (the *meta*- or extradiol-cleavage) (Smith, 1990). The simplest of substituted benzenes is **toluene** representing the mono-alkylbenzenes (Smith, 1990). ***m*-, *p*- and *o*-xylenes** belong to the di-alkylbenzenes (Smith, 1990). Benzene, toluene, ethyl benzene, *m*- and *p*-xylene are usually referred to as **BTEX compounds**. They are known to be degraded by bacterial enzymes encoded on TOL plasmids (Assinder & Williams, 1990). **Biphenyl** may be considered as substituted benzene, the substituent being benzene itself (Smith, 1990). **Phenol**, i.e. phenyl alcohol or carbolic acid, is an alcohol and a weak acid.

Among the most recalcitrant petroleum compounds are **POLYCYCLIC AROMATIC HYDROCARBONS** (**PAHs**), which comprise a wide range of hydrophobic compounds with two or more aromatic benzene rings (Chaudhry et al., 2005). The simplest PAH compounds are **naphthalene** and 2-bromo-naphthalene with two benzene rings (Smith, 1990). Acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene are 3-ring PAHs; fluoranthene, pyrene, chrysene and benzo(a)anthracene are 4-ring PAHs; benzo(b)fluoranthene, benzo(a)pyrene and dibenzo(a,h)anthracene are 5-ring PAHs; and indeno(1,2,3-c,d)pyrene and benzo(g,h,i) perylene are 6-ring PAHs.

1.2.2 Genes and plasmids involved in aerobic biodegradation of monoaromatics

BTEX compounds are aerobically degraded via catechol and subsequently the *meta* pathway by several strains of *Pseudomonas* by enzymes encoded on plasmids, designated **TOL** (Assinder & Williams, 1990). These plasmids contain two **catabolic operons** (Fig. 1-4). The **'upper' pathway** operon (*xylMABCXYZ*) encodes enzymes for the successive oxidation of the hydrocarbons to the corresponding alcohol, aldehyde and carboxylic acid derivatives. The **'lower' or *meta*-cleavage pathway** operon (*xylLEFGHIJK*) encodes enzymes for the conversion of the carboxylic acids to catechols, whose aromatic rings are then cleaved (*meta*-fission) to produce corresponding

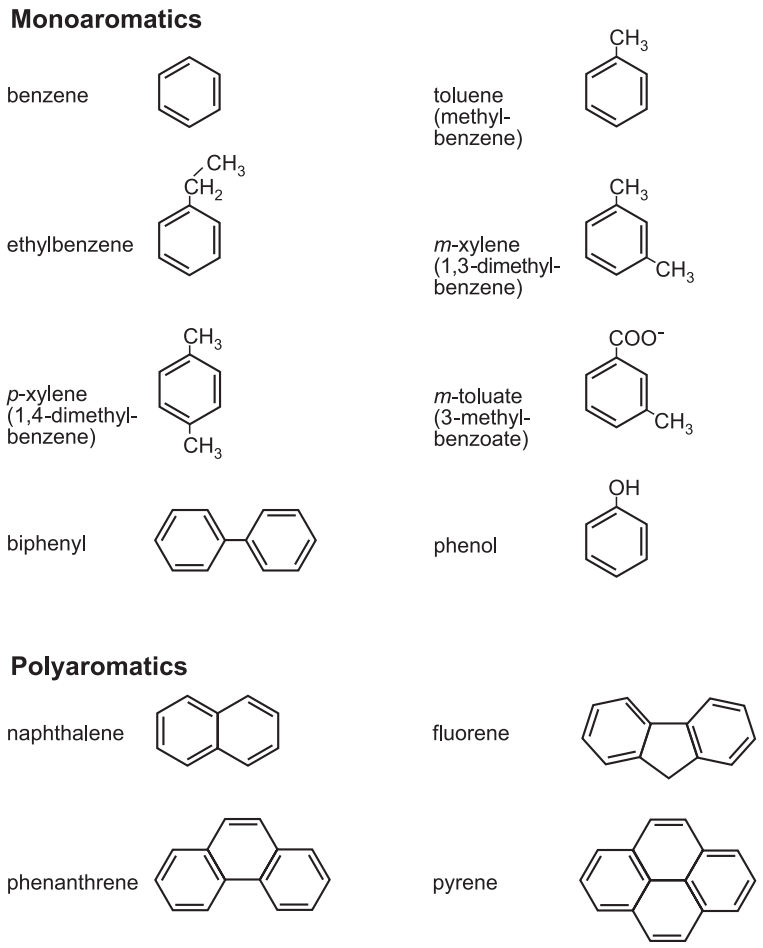


Fig. 1-3. Chemical structure of some aromatics.

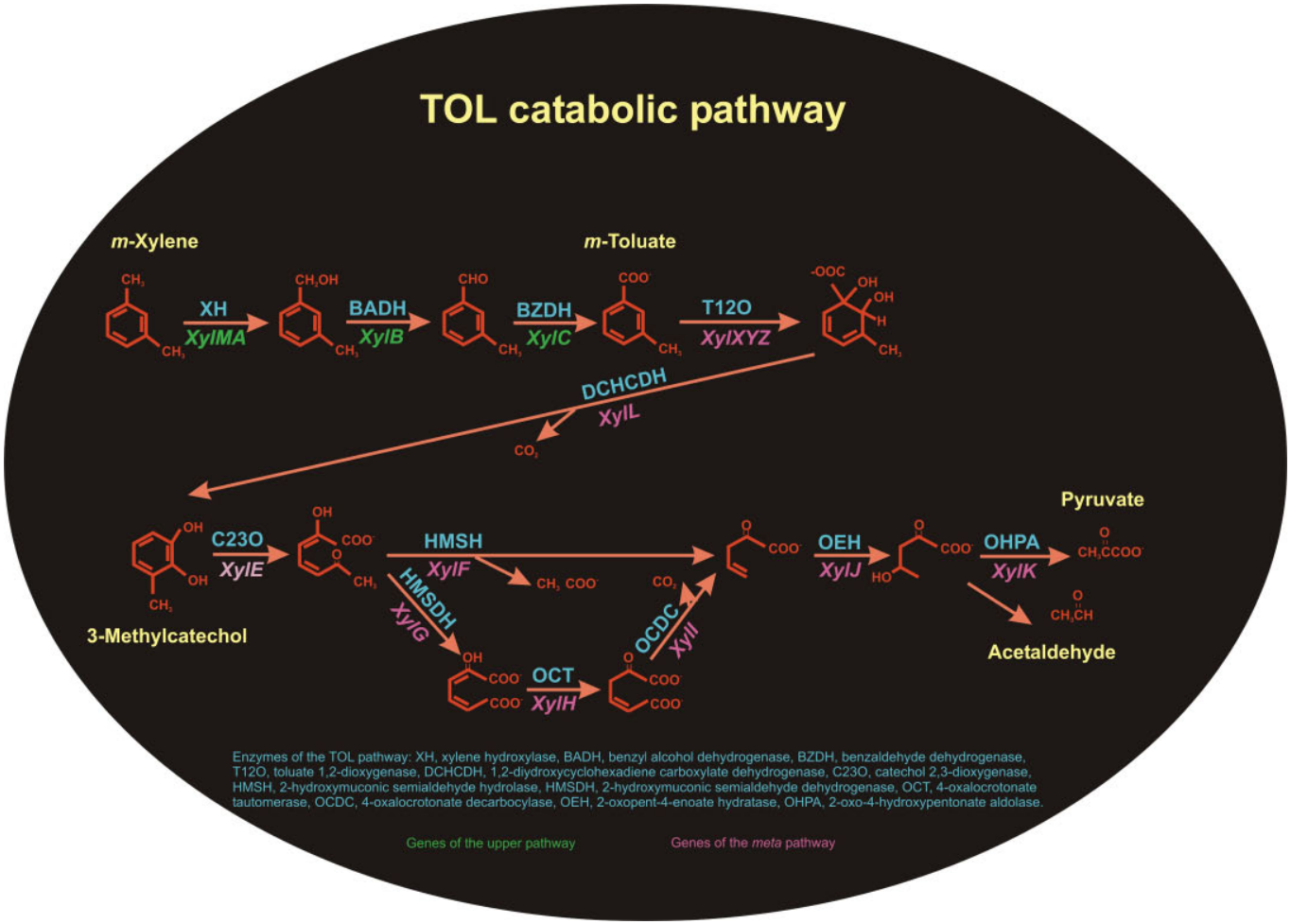


Fig. 1-4. TOL plasmid encoded catabolic pathway. The upper pathway on top and the *meta* pathway below. Illustration is based on Houghton & Shanley (1994).

semialdehydes, which are then further catabolized through the tricarboxylic acid (TCA) cycle. The *xyl* genes located on TOL plasmids reside within transposable elements (Assinder & Williams, 1990). The *meta*-cleavage pathway on TOL plasmids differs significantly from the chromosomal **β-ketoadipate or *ortho*-cleavage pathway** in being able to tolerate alkyl substituents on the catechol (Assinder & Williams, 1990). This enables TOL-harboring bacteria to utilize not only benzoate but also ***m*-toluate** (3-methylbenzoate) and *p*-toluate (4-methylbenzoate), which are converted to respective alkylcatechols. The most studied, **archetypal** TOL plasmid **pWW0** (117 kb) was found in *P. putida* mt-2 (Willams & Murray, 1974), a strain originally isolated in Japan in the 1950s (Keil et al., 1987). The majority of TOL plasmids identified have been shown to bear strong DNA sequence homology to the archetypal TOL plasmid pWW0 (Houghton & Shanley, 1994).

The gene ***xylE*** codes for catechol 2,3-dioxygenase (**C23O**), which is one of the KEY ENZYMES in the *meta*-cleavage pathway on TOL plasmids (Houghton & Shanley, 1994). The breakdown of catechol – one of the central catabolic intermediates – by C23O can be detected as a yellow product called 2-hydroxymuconic semialdehyde (Assinder & Williams, 1990; Houghton & Shanley, 1994). However, the indirect detection of C23O from bacteria based only on the phenotype does not allow the detection of C23Os from yellow-pigmented bacteria such as *Sphingomonas* and *Arthrobacter* or the detection of silent or inactive C23O genes.

Many CATABOLIC PATHWAYS are carried ON PLASMIDS (Sayler et al., 1990). The pathway for the catabolism of toluene (*xyl*) shares many features with the catabolic pathways of other aromatic compounds like biphenyl (*bph*), naphthalene (*nah*) and phenol (*dmp*) (Carrington et al., 1994). The genes of these pathways are situated on plasmids **TOL**, **BPH**, **NAH** and **PHE**, respectively, and each pathway produces a **C23O** belonging to the **subfamily A of the** extradiol aromatic ring-cleavage dioxygenase (**EDO**) **family I.2**. Thus, these four pathways converge at benzoate, which is assimilated *via* catechol and subsequent *meta*-cleavage in some aromatic degrading bacteria (Assinder & Williams, 1990; Houghton & Shanley, 1994).

Most of the degradation plasmids are conjugative, i.e. self-transmissible, but the host range maintenance varies and thus, these plasmids have a restricted potential to spread to other bacteria in the soil (Assinder & Williams, 1990; Sayler et al., 1990). Ecologically, plasmid-encoded pathways are advantageous because they provide genetically flexible systems and can be maintained in the population and transferred between bacterial species (Sayler et al., 1990). **Bacterial conjugation** is the most widespread mechanism for **HORIZONTAL GENE TRANSFER** (**HGT**) with potential for universal DNA delivery (Llosa & de la Cruz, 2005). Any specific gene in the extended gene pool outside the chromosome is maintained in the population by just a few cells, thus consuming little energy from the population as a whole, but is ready to spread upon selective pressure such as oil contamination (Llosa & de la Cruz, 2005).

1.2.2.1 Aromatic ring-cleavage dioxygenases

The biodegradation of aromatic rings play a major role in the maintenance of the global carbon cycle (Bugg & Winfield, 1998). Dioxygenases, which catalyze the incorporation of both atoms of dioxygen into their substrates, are widely distributed in nature and are involved in both anabolic and catabolic processes (Eltis & Bolin, 1996). One important catabolic strategy used by bacteria is the aerobic degradation of aromatic compounds where dioxygenases catalyze two critical reactions, ring dihydroxylation and ring cleavage. A typical substrate of the latter reaction is a catecholic metabolite possessing hydroxyl substituents on two adjacent carbon atoms. Cleavage is generally catalyzed by metalloenzymes of one of two functional classes. **Intradiol** aromatic ring-cleavage dioxygenases cleave *ortho* to the hydroxyl substituents (between two hydroxyl groups) and typically depend on nonheme Fe(III). In contrast, **EXTRADIOL AROMATIC RING-CLEAVAGE DIOXYGENASES** (**EDO**) cleave *meta* to the hydroxyl substituents (between one hydroxyl carbon and its adjacent nonhydroxylated carbon) and typically depend on nonheme Fe(II) (Eltis & Bolin, 1996).

Extradiol dioxygenases can be divided phylogenetically into two types (Eltis & Bolin, 1996). **Type I extradiol dioxygenases** can be further divided into five families. The 2,3-

dihydroxybiphenyl 1,2-dioxygenase of *Rhodococcus globerulus* P6 coded by the gene *bphC* is an example of the family I.1, which comprises the single-domain enzymes. However, most BphC enzymes from both gram-negative and gram-positive bacteria are classified into the family I.3. The families I.2 and I.3 consist of two-domain, iron-containing enzymes that show preferences for monocyclic and bicyclic substrates, respectively. The genes coding for the enzymes of the SUBFAMILY A OF THE FAMILY **I.2** are closely related, they are found in *Pseudomonas* species degrading aromatics, and they produce **C23Os** which have preference for monocyclic substrates (Carrington et al., 1994; Eltis & Bolin, 1996). One of these genes is the ***xylE*** gene on the TOL plasmid pWW0. Catechol 2,3-dioxygenase MpcII of *Alcaligenes eutrophus* JMP222 belongs to the family I.4 and Mn-dependent 3,4-dihydroxyphenyl-acetate 2,3-dioxygenase MndD of *Arthrobacter globiformis* CM-2 to the family I.5. **Type II extradiol dioxygenases** are divided into two families II.1 and II.2, and protocatechuate 4,5-dioxygenase of *Pseudomonas paucimobilus* and catechol 2,3-dioxygenase I of *A. eutrophus* represent them, respectively (Eltis & Bolin, 1996).

1.2.3 Composition of oil-degrading bacterial communities in the rhizosphere

Although the importance of the rhizosphere community for degradation of pollutants has been recognized, very little is known about the survival, proliferation, activity, and exact composition of degrading populations in the rhizosphere (Kuiper et al., 2004). BACTERIAL DIVERSITY in general is an aspect of the rhizosphere effect that has got little attention in rhizoremediation studies thus far. The composition of the microbial populations in the rhizosphere may vary with plant species, the composition of the root exudate, root type, plant age, soil type, the history of the soil, environmental factors, and the pollutant (Anderson et al., 1993; Kuiper et al., 2004; Chaudhry et al., 2005).

The microbial populations in a plant rhizosphere are usually composed of DIVERSE AND SYNERGISTIC communities (Chaudhry et al., 2005). Such diversity further promotes remediation of contaminated soils as degradation of organic compounds may require several organisms with distinctive enzyme systems. The exudation of certain compounds by plant roots can enhance the growth of specific pollutant-degrading microbes in a rhizosphere, e.g. PAH degraders (Günther et al., 1996; Joner et al., 2002) though the proliferation may be non-selective (da Silva et al., 2006). Siciliano et al. (2003) showed that the hydrocarbon degrading potential of rhizosphere soil increased through changes in the composition of a microbial community towards those containing the degradative genes *ndoB*, *alkB*, and *xylE*. The composition of microbial community in a rhizosphere is also known to differ both QUALITATIVELY AND QUANTITATIVELY from that in a non-rhizospheric soil (Chaudhry et al., 2005). Beyond the rooting zone, the microbial growth-stimulating effects of exudates seemed to diminish rapidly (Badalucco et al., 1997). Larger populations, and selective enrichment, of organic compound degrading microorganisms have been found in the rhizospheres of alfalfa and bluegrass compared to non-contaminated soils (Nichols et al., 1997). Rhizospheres of certain desert and crop plants grown in oil-polluted soils have been shown to contain more hydrocarbon-utilising bacteria, such as *Cellulomonas flavigena*, *Rhodococcus erythropolis* and *Arthrobacter* sp., than in control soils (Radwan et al., 1998). Greater activities of denitrifiers, pseudomonads, and BTEX-degrading microbes have been reported in the rhizosphere of poplar trees than in adjacent soils (Jordahl et al., 1997).

Observations have supported the fact that the rhizosphere is dominated by gram-negative rods, such as *Pseudomonas*, *Rhizobium* and *Azotobacter*, probably due to efficient utilization of growth substrates and detoxifying enzymes produced (Chaudhry et al., 2005; Kuiper et al., 2004). However, these observations may be biased due to limitations of (culturing) techniques to reveal the real situation in nature. For example, only up to 1% of soil microbial species can currently be cultured in the laboratory (Chaudhry et al., 2005; Kirk et al., 2004). Liste & Prutz (2006) identified a variety of bacteria, both gram-negative and gram-positive, from PHC-contaminated rhizosphere, expressing aromatic ring dioxygneases on indole agar (Table 1-1). Some of these species were typical for pristine, PHC-contamintated or both soils. Isolates identified from the rhizoremediation plant *Cyperus laxus* have belonged to genera *Achromobacter* (*Alcaligenes*),

Bacillus, *Brevibacterium*, *Pseudomonas*, *Kocuria*, *Gordonia*, *Arthrobacter* and *Micrococcus* (Díaz-Ramírez et al., 2003; Medina-Moreno et al., 2005). However, systematic identification of CULTURABLE DEGRADATION BACTERIA in the rhizosphere has still been missing.

AT COMMUNITY LEVEL, the analysis of the structure and function of bacterial populations is based usually on DNA (e.g. DGGE) and physiological profiles (e.g. CLPP), respectively. The composition of a microbial community has been observed to change in a soil contaminated with DBT-containing petroleum with time favouring those species that were specialised in degrading DBT (*Rhodococcus erythropolis*) shown by 16S rRNA-DGGE method (Duarte et al., 2001). The changes in the physiological structure of the microbial community under bitumen contamination were hinged on not merely the presence of plants but also their type (Muratova et al., 2003). Kirk et al. (2005) detected no changes in metabolic diversity (CLPP with Eco-Biolog) but a shift in the bacterial community in the PHC-contaminated rhizosphere (16S rDNA-DGGE). On the contrary, Siciliano et al. (2003) found no detectable shift in the 16S rDNA composition between treatments but there were plant-specific and -selective effects on specific catabolic gene prevalence. More relevant information might be reached by focusing the DGGE analysis on few but important physiological groups of bacteria or on a group of specific degradation genes.

1.2.4 Effective biodegradation of petroleum hydrocarbons in the rhizosphere

A number of studies suggest that microbial strains capable of breaking down petroleum hydrocarbons are widely present among soil microorganisms (Chaudhry et al., 2005). Some PLANT SPECIES seems to SELECT AND ENRICH these strains in the rhizosphere soils. For example, less inhibition and more degradative potential of the rhizosphere microflora of alfalfa (*Medicago sativa*) has been shown compared to that of reed plants (Muratova et al., 2003). In addition, the total hydrocarbons in a diesel-contaminated soil decreased faster in the presence of roots and nutrients compared to that in non-vegetated or non-fertilized soils (Reynolds et al., 1999; Yateem et al., 2000). An optimal addition of fertilizers may be important to enhance breakdown of diesel compounds like it was observed in the rhizosphere of some grass and legume species (Pichtel & Liskanen, 2001).

Table 1-1. Occurrence of aromatic ring-oxidizing bacteria species in petrol hydrocarbon-contaminated (PHC) and uncontaminated (PRI) soil. Table modified from Liste & Prutz (2006).

Bacteria			**Occurrence**	
Genus	**Species**	**GC subgroup**	**PHC**	**PRI**
Bacillus	*megaterium*	A	-	+
	megaterium	B	-	+
	simplex		-	+
	subtilis		-	+
Brevibacillus	*brevis*		-	+
Cellulomonas	*cartae*		+	+
	turbata		+	+
Comamonas	*acidovorans*		+	-
Kocuria	*kristinae*		-	+
	varians		-	+
Microbacterium	*esteraromaticum*		-	+
Micrococcus	*luteus*	C	-	+
Pseudomonas	*balearica*		+	-
	stutzeri		+	-
Staphylococcus	*warneri*		-	+
Stenotrophomonas	*maltophilia*		+	-

Global interest for rhizoremediation can be seen in the research flourishing in the 21st century (Table 1-2). The amount of field trials is increasing to document efficient rhizoremediation also in naturally fluctuating conditions. Several grasses and legume plants have been the most studied species for the rhizoremediation of various forms of petroleum hydrocarbons. However, the study of monoaromatics has largely been ignored. Volatile hydrocarbons have even been evaporated before the study of more persistent compounds (Muratova et al., 2003). Robson et al. (2003) considered that screening plants for hydrocarbon tolerance before screening for degradation ability may prove more economical than screening directly for degradation. Some tropical legumes, for instance, were not tolerant of crude oil long enough but a hardy tropical grass *Brachiaria brizantha* caused a considerable reduction of aromatics (Merkl et al., 2005b).

The rhizosphere influences bacteria in several ways, which play important roles in rhizoremediation. In addition to the increased BACTERIAL ABUNDANCE (e.g. Kaimi et al., 2006; Escalante-Espinosa et al., 2005), the rhizoremediation studies have most often documented the increased amount of DEGRADATION BACTERIA in the rhizosphere (e.g. Kirk et al., 2005; Tang et al., 2004) leading to enhanced degradation (e.g. White et al., 2006; Liste & Prutz, 2006) though the degradation differences between planted and unplanted soils may diminish by time (Merkl et al., 2005a). The enhanced degradation of aliphatic hydrocarbons during growth of ryegrass (*Lolium perenne*) has been associated with an increase in microbial NUMBERS AND ACTIVITIES in the rhizosphere compared to that in bulk soil (Günther et al., 1996). Kaimi et al. (2006) found that ryegrass roots were effective in enhancing the biodegradation of diesel-contaminated soil while the soil dehydrogenase activity was high and also correlated with the growth of roots. The level of CATABOLIC GENES measured by Q-PCR has lately been adapted to describe the degradation activity in the rhizosphere (e.g. Siciliano et al., 2003; da Silva et al., 2006). Instead, the DYNAMICS OF GENES in the context of rhizoremediation has been ignored thus far.

The enhancement of oil degradation, however, has not always been clearly correlated to microbial numbers and activity. Merkl et al. (2006) suggested OTHER FACTORS like oxygen availability, plant enzymes, and synergistic degradation by microbial consortia (bacteria-bacteria; bacteria-fungi) to be considered. Plant organic matter had an impact on PAH attenuation in both labile and refractory fractions of petroleum distillate waste (Gregory et al., 2005). Plants may, thus, enhance the bioavailability of initially unextractable molecules (Liste & Prutz, 2006). Similarly, faster degradation of PAHs has been reported in vegetated soils than in non-vegetated soils (Reilley et al., 1996; Aprill & Sims, 1990; Daane et al., 2001) and this has been mediated by root-associated microorganisms (Anderson et al., 1993; Radwan et al., 1998). Many bacterial species are able to use PAHs of low and intermediate molecular weights as a sole source of carbon (Aitken et al., 1998; Bouchez et al., 1999) but degradation of high molecular weight PAHs needs co-oxidation in the presence of a more readily degradable carbon source (Cerniglia, 1997). Table 1-3 shows some of the recent studies on enhanced biodegradation of petroleum compounds in NON-RHIZOSPHERIC SOIL. These studies enlighten the role of separate factors such as nutrients (biostimulation) and bacterial consortia (bioaugmentation) in bioremediation.

PRE-EXPOSURE to a particular pollutant may enhance the degradation potential of soil/rhizosphere microorganisms upon re-exposure to the same compound (Chaudhry et al., 2005). This is due to the growth of selected microbial populations that may retain a specific metabolic capability over long periods of time, a phenomenon known as '**soil memory**'. For instance, mineralisation of *p*-nitrophenol by microorganisms in rice rhizosphere was faster in both planted and unplanted soil systems that were pre-exposed to the compound, compared to a soil without pre-exposure (Reddy & Sethunathan, 1994).

1.3 Monitoring of microbial communities in chemically contaminated soil – limitations and potentials

SOIL is a very demanding environment to study because of the high HETEROGENEITY with its changing temporal and spatial microhabitats for microorganisms (Tate, 1995; Kozdrój & van Elsas, 2001; Kirk et al., 2004). Therefore in soil microbiology, the first BIASES to results appear at the stage of soil SAMPLING and STORAGE (Kozdrój & van Elsas, 2001; Kirk et al., 2004).

Table 1-2. Recent studies on enhanced biodegradation of petroleum hydrocarbons in the rhizosphere.

Plant	Pollutant	Type of experiment; Location	Results	Reference
ryegrass *Lolium multiflorum*	diesel oil	GH; Japan	The residual rate of diesel oil in the rhizosphere was 55% lower than in the corresponding root-free soil, and the dissipation threshold reduction occurred after the development of plant roots. In the rhizosphere, the number of aerobic bacteria and the amount of soil dehydrogenase activity were higher than in the root-free soil and also showed a correlation with the growth of roots. The dissipation rate of diesel oil showed a correlation with soil dehydrogenase activity in both the rhizosphere and the root-free soil. A positive correlation was observed between the growth rate of roots and soil dehydrogenase activity in the rhizosphere. Moreover, the dissipation rate per dehydrogenase activity of the rhizosphere was higher than in the root-free soil. **Ryegrass** roots were effective at **enhancing** the **biodegradation** of diesel-contaminated soil.	Kaimi et al., 2006
grasses, cruciferes, legumes, herbs	PHC-contaminated soil from former manufactured gas plant	GH; Germany	Total bacteria and aromatic ring dioxygenase-expressing bacteria were more abundant in contaminated soil and were most numerous in the rhizosphere of white mustard (*Sinapis alba*). The loss of TPHs and PAHs was greatest with hemp (*Cannabis sativa*) and white mustard. Pea (*Pisum sativum*), cress (*Lepidium sativum*) and pansy (*Viola tricolor*) increased the amounts of PAHs extracted from soil. **Plants** may **enhance** the chemical extractability and perhaps **biological availability** of initially unextractable molecules.	Liste & Prutz, 2006
tropical pasture grass *Brachiaria brizantha*	5% of a heavy crude oil with 40% aromatics	GH; Venezuela	Mostly increasing effect on microbial numbers but the growth of bacteria was not or negatively affected. Microbial respiration and pH lower in planted soil. Since fungi tolerate lower pH values than bacteria, they are considered to play a central role in oil degradation. Low pH may result from enhanced oil degradation in planted soil leading to an accumulation of organic acids. A comparable stimulation of crude oil degraders and fungi. Because the **enhancement** of crude oil **degradation** did **not** clearly **correlated to microbial numbers and activity**, **other factors** like oxygen availability, plant enzymes and synergistic degradation by microbial consortia have to be considered.	Merkl et al., 2006
mulberry + root extracts (phenolics)	aged phenanthrene (PAH)	GH; Texas, USA	Exposure to root extracts enhanced the growth of total bacteria and PAH degraders (*nahAc*, *todC1*, *bmoA*, *dmpN* by Q-PCR) in both contaminated and uncontaminated rhizosphere. The relative abundance of PAH-degrader gene copies (as a fraction of the total bacteria) was similar for different treatments, suggesting that the root extracts did not select for PAH degraders. Rhizodeposition from phenolic releasers contributed to the **fortuitous, but not selective, proliferation of PAH degraders, which may enhance phytoremediation**.	da Silva et al., 2006
fescue *Lolium arundinaceum* - ryegrass *L. multiflorum* mixture, bermudagrass *Cynodon dactylon* - fescue mixture	crude oil	FE; Arkansas, USA	Greater degradation of three-ring alkylated phenanthrenes-anthracenes and DBTs in the vegetated fertilized plots. An increase in rhizosphere soil volume associated with increased root length along with nutrient additions resulted in increased total bacterial, fungal, and PAH degrader numbers. **Increased degrader numbers** in the rhizosphere most likely resulted in **increased biodegradation** of the more recalcitrant alkylated PAH compounds.	White et al., 2006
Cyperus laxus and its PHC-degrading rhizosphere consortium (10 bacteria and 3 fungi)	TPH	GH; Mexico	Phenological charcteristics of inoculated plants were improved: greater root biomass, reduced flowering time, more inflorescences. The rhizospheric bacterial and fungi counts were higher for planted treatments, both inoculated and not inoculated, than for unplanted pots. The maximum phytoremediation rate for inoculated plants was reached at 60 days of culture, and was two times higher than for non-inoculated plants. Similar PHC phytoremediation extent values for inoculated and non-inoculated plants were obtained at 180 days of culture. **The mutual benefits between *C. laxus* and inoculated PHC-degrading microorganisms were improved during phytoremediation**.	Escalante-Espinosa et al., 2005

Table 1-2 *(continued)*

Phragmites australis, *Phragmites* with goldenrod *Solidago* sp. and switchgrass *Panicum* sp., tree of heaven *Ailanthus altissima* and bayberry shrubs *Myrica* sp.	a petroleum distillate waste pit (PAHs)	FE; North Carolina, USA	Inputs of biogenic plant carbon, PAH weathering, and declines in PAH concentrations were most evident for vegetated SOM fractions, particularly humin fractions. Sequestered PAH metabolites were also observed in vegetated humin. **Plant organic matter impacted PAH attenuation** in both labile and refractory fractions of petroleum distillate waste.	Gregory et al., 2005
perennial ryegrass *Lolium perenne* and/or alfalfa *Medicago sativa*	TPH	GH; Canada	The number of rhizosphere bacteria increased. The number of bacteria capable of petroleum degradation increased (MPN method). No changes in metabolic diversity (Eco-Biolog) but a shift in the bacterial community in the rhizosphere (16S rDNA-DGGE). **Plants altered plant-specifically the microbial population**. These changes **could contribute to degradation** of petroleum hydrocarbons.	Kirk et al., 2005
tropical legumes and grasses	5% of a heavy crude oil with 40% aromatics	GH; Venezuela	The legumes died within six to eight weeks. The grasses showed reduced biomass production. Relative growth rates were higher in contaminated soil indicating a delay in plant growth patterns and development. Soils with the grasses *Brachiaria brizantha* and *Cyperus aggregatus* showed a significantly lower oil concentration than non-vegetated soil. A positive correlation between root biomass production and oil degradation. Concentration of saturated hydrocarbons lower in planted soil. ***B. brizantha* caused a considerable reduction of aromatics**. *B. brizantha* was recommended for follow-up investigations which could further develop the application of phytoremediation of petroleum-contaminated soils in the tropics.	Merkl et al., 2005b
tropical pasture grass *Brachiaria brizantha*	5% of a heavy crude oil with 40% aromatics	GH; Venezuela	The medium NPK-fertilizer concentration resulted in best root growth and highest absolute oil dissipation (18.4%) after 22 wk. The highest NPK concentration produced best shoot growth and highest relative oil dissipation after 14 wk. **Degradation** of total oil and grease was **higher in planted** than in unplanted soil, but differences diminished toward the end of the experiment. Next to **fertilizer quantity, its composition** is an important factor to be further studied. Field trials would be indispensable for further phytoremediation studies, since greenhouse experiments produce particular water and nutrient conditions.	Merkl et al., 2005a
white mustard *Sinapis alba* and soil isolate	phenanthrene (PAH)	LAB; Canada	The soil bacterium *Sphingomonas yanoikuyae* was isolated from a PHC-contaminated soil. The gas phase concentration of phenanthrene in nonsterile sand decreased by 99.7% with *S. alba* plus *S.y.*, by 98.6% with *S.a.*, by 96.7% with *S.y.*, and by 95.8% with no additions. Under gnotobiotic conditions, the gas phase concentration of phenanthrene in sand decreased by 94% with *S. alba* plus *S. y.*, by 77% with *S. y.*, by 26% with *S. alba.*, and by 0% with no additions. Solid-phase-microextraction (SPME) gas chromatography - flame ionization detection (GC-FID) could be used to rapidly assess the **potential of plants and microorganims to reduce** the level of unaged PAHs such as phenanthrene in soil.	Hynes et al., 2004
orchad grass *Dactylis glomerata* with filter technology (permeable barriers)	creosote (PAHs, NSO-heterocyclic compounds and phenols)	LAB; Norway	The combination of sorption and microbial processes led to an efficient disappearance of all analyzed compounds from the water, where biological processes were dominant for removal of phenols. Vegetated filter material was the most efficient in treating the contaminated water and the largest effect of plant was observed during periods where water residence time was short. A soil/ sand filter material could efficiently treat creosote-contaminated groundwater, whereas the presence of **orchard grass further improved treatment**.	Rasmussen & Olsen, 2004
tropical trees	diesel-fuel (PAHs and alkanes)	GH; Hawaii, USA	Milo (*Thespesia populnea*) and kou (*Cordia subcordata*) were more effective than false sandalwood (*Myoporum sandwicense*) in reducing the concentration of the spiked contaminant. Enumerations of populations of PHC-degrading microorganisms in the bottom section suggested that biodegradation may be affected by the response of microorganisms to both the “close rhizosphere” (soil within 1 mm of the root) and the “expanded rhizosphere” (soil in the bottom section after root removal). **Root exudates** leached from the upper sections could be **responsible for the expanded rhizosphere effect** in the bottom section.	Tang et al., 2004
alfalfa *Medicago sativa*, reed *Phragmites* australis	bitumen	GH; Germany	Bitumen reduced the total number of microorganisms more significantly in unplanted soil and had various effects on some important physiological groups of microorganismis. The changes in the **physiological structure of the microbial community** under bitumen contamination were found to **hinge on** not merely the presence of **plants** but also their **type**. The rhizosphere microflora of **alfalfa** was less inhibited by hydrocarbon pollution and had a **higher degradative potential** than the rhizosphere microflora of reed.	Muratova et al., 2003

Table 1-2 *(continued)*

grasses and legumes	aged diesel fuel (PAHs)	FE; California, USA	In the bulk soil, the level of catabolic genes involved in PHC degradation (*ndoB*, *alkB*, *xylE* by probing) as well as the mineralization of hexadecane and phenanthrene was higher in planted treatment cells. There was no detectable shift in the 16S rDNA composition (DGGE) of the bulk soil community between treatments, but there were plant-specific and -selective effects on specific catabolic gene prevalence. Tall fescue (*Festuca arundinacea*) increased the prevalence of *ndoB*, *alkB*, and *xylE* as well as naphthalene mineralization in rhizosphere soil. In contrast, rose clover (*Trifolium hirtum*) decreased catabolic gene prevalence and naphthalene mineralization in rhizosphere soil. **Phytoremediation systems increased the catabolic potential of rhizosphere soil by altering the functional composition of the microbial community**. This change in composition was not detectable by 16S rDNA but was **linked to specific functional genotypes with relevance to PHC degradation**.	Siciliano et al., 2003

Pollutant: AF-TPH, asphaltene-free total petroleum hydrocarbons; BTEX, benzene/toluene/ethylbenzene/xylenes; DBT, dibenzothiophene; PAH, polycyclic aromatic hydrocarbon; PHC, petroleum hydrocarbons; TPH, total petroleum hydrocarbons; Type of experiment: FE, field; GH, greenhouse; LAB, laboratory; Method: CLPP, community level physiological profile; DGGE, denaturant gradient gel electrophoresis; MPN, most probable number; Q-PCR, real time quantitative PCR; SOM, soil/sediment organic matter; Gene - coding for: *alkB*, alkane monooxygenase, *bmoA*, hydroxylating monooxygenase; *dmpN*, phenol hydroxylase; *nahAc*, naphthalene dioxygenase; *ndoB*, naphthalene dioxygenase; *todC1*, toluene/benzene/chlorobenzene dioxygenase; *xylE*, catechol 2,3-dioxygenase; Other: NPK, nitrogen/phosphorous/kalium; NSO, nitrogen/sulfur/oxygen.

Table 1-3. Recent studies on enhanced biodegradation of petroleum hydrocarbons in non-rhizospheric soil. For abbreviations see Table 1-2.

Enhancer	Pollutant	Type of experiment; Location	Results	Reference
nutrient amendments	paraffinic (crude) oil with 26% aromatics	GH; France	The maximal biodegradation extent was 62%. The natural attenuation contributed to 47% of degradation. A permanent inhibition of hydrocarbons assimilation was recorded with a high input of nutrients. The biodegradation of saturates, aromatics and polars was respectively, permanently, temporally and not reduced by excessive fertilization in soil. Accumulation of polar metabolic by-products. Maximum stimulation of growth of total heterotrophic bacteria and hydrocarbon-adapted bacteria was observed with the highest input of nutrients. However, the extents of biodegradation were not concurrently improved indicating that the microbial degraders were selected depending on the nutrient supply. The permanent and/or temporally inhibition of the saturated and unsaturated hydrocarbons assimilation revealed that **different nutrient supplies were optimum for the degradation of aliphatic and aromatic hydrocarbons**.	Chaîneau et al., 2005
a consortium from the rhizosphere of *Cyperus laxus*	crude oil – paraffin mixture, AF-TPH	LAB; Mexico	A consortium isolated from a native plant that grows naturally in weathered, PHC-contaminated sites. The extent and biodegradation rates increased significantly up to the fourth cycle. Thereafter, constant general trend of biodegradation. The effect of oxygen limitation on consortium activity led to a low biomass yield and non-soluble metabolite. The average number of PHC-degrading microorganisms increased ten-fold to the fourth cycle. Five bacterial strains were identified: *Achromobacter* (*Alcaligenes*) *xylosoxidans*, *Bacillus cereus*, *B. subtilis*, *Brevibacterium luteum*, and *Pseudomonas pseudoalcaligenes*. AF-TPHs were also biodegraded and mineralized by the enriched consortium without inhibition. **Sequential batch reactors under oxygen limitation** can be used **to produce consortia with high and constant biodegradation ability** for industrial applications of bioremediation.	Medina-Moreno et al., 2005
natural attenuation in soil	PHC-mixture: artificial kerosene (jet-fuel) containing BTEX	FE; Denmark	Total cell numbers (fluorescence microscopy) were strongly correlated with soil organic carbon and nitrogen content but varied little with contamination. Variation in biomass and CO_2 production was explained by soil parameters, to 46%, and by the duration of contamination, to 39.8%. In the CLPP data (Biolog^{TM} Eco-Plates) only 35.9% of the variation could be linked to soil parameters and contamination, however, the samples with greatest exposure to hydrocarbons grouped together on redundancy analysis (RDA) plots. **At nutrient-poor site, the microbial community was dominated by natural heterogeneity and the influence of PHC vapours was weak**.	Kaufmann et al., 2004
10 isolates and a consortium from *Cyperus laxus* rhizosphere	crude oil	GH; Mexico	Five bacterial strains were able to degrade more than 50% of the aliphatic fraction. The most extensive biodegradation (74%) was obtained with *Bacillus subtilis* 9A, while *Pseudomonas* sp. 2AP and unidentified strain UAM 10AP were able to degrade up to 15% of the aromatic-polar mixture. The defined mixture culture degraded 47% of the aliphatic fraction and 26% of the aromatic fraction when grown in the presence of TPHs, while these microorganisms did not consume the polar fraction. Predominant, identified bacteria with the aromatic-polar mixture were *B. cereus*, *P.* sp., *Kocuria rosea* and *B. subtilis*. In addition to these, predominant bacteria with aliphatics were *Gordonia rubripertincta*, *Arthrobacter oxydans* and *Micrococcus luteus*. Alipahtics degrading bacteria represented 10% of the total bacterial population observed in the rhizosphere of *C. laxus*, while the number of aromatics degrading bacteria corresponded to 3% of the total bacterial count. The strategy that combines enrichment culture together with oxygen uptake rate allowed **the isolation of bacterial strains** that were able **to degrade specific PHC fractions** at high consumption rates.	Díaz-Ramírez et al., 2003

Soil microorganisms, such as bacteria and fungi, play central roles in soil fertility and promoting plant health (Kirk et al., 2004). In addition, the ecological role of Archaea in MICROBIAL COMMUNITIES inhabiting also usual environments, such as boreal soil, has only recently started to be revealed (Jurgens, 2002). However, some of the characteristics of microorganisms cause LIMITATIONS in studying microbial diversity. First, we are UNABLE TO CULTURE all soil microorganisms since it is estimated that only 1% of the soil bacterial populations can be cultured by standard laboratory practices (Kirk et al., 2004). Earlier the study of microorganisms in natural environments was mostly based on cultivation and isolation techniques (Kozdrój & van Elsas, 2001; Lynch et al., 2004). The limits of these methods have been frequently discussed (e.g. Torsvik et al., 1996). Second, microbes are TAXONOMICALLY AMBIGUITOUS. The genetic plasticity of bacteria, allowing DNA transfer through plasmids, bacteriophages and transposons, complicates the concept of bacterial species (Kirk et al., 2004). However, the basic phylogeny of bacteria can be based on the rRNA gene (16S or 23S) because this gene is present in all bacteria, it has well defined regions for taxonomic classification that are not subjected to horizontal transfer and sequence databases are available for researches (Kirk et al., 2004; Lynch et al., 2004).

Both traditional, **BIOCHEMICAL-BASED** and modern, **MOLECULAR-BASED APPROACHES** can be used FOR MONITORING of microbial communities in contaminated soil (Fig. 1-5). Advantages and disadvantages of some biochemical methods are presented in Table 1-4. The focus in following chapters is, however, on the molecular approaches. When COMBINED with classic microbiological methods, molecular biological methods could provide us with a more comprehensive interpretation of the *in situ* microbial community and its response to bioremediation processes (Widada et al., 2002).

Microbial ecology studies have benefited from the use of molecular methods, which allow the detection of both culturable and unculturable microbial species. Despite these advances in the field of **MOLECULAR ECOLOGY**, the LINK BETWEEN MICROBIAL DIVERSITY AND SOIL FUNCTIONS is still a major challenge (Lynch et al., 2004). Changes in the composition of soil microflora can be crucial for the functional integrity of soil (Insam, 2001; Lynch et al., 2004). The interrelationship between

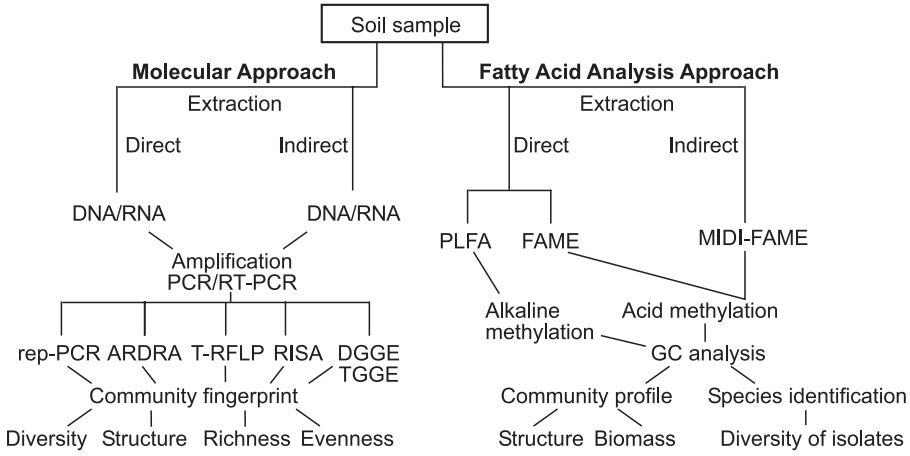


Fig. 1-5. Approaches available for microbial community fingerprinting in chemically perturbed soil. Figure adapted from Kozdrój & van Elsas (2001). These approaches are based on the analyses of signature biomarkers, i.e. indicator molecules, such as nucleic acids and fatty acids. Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; DGGE, denaturant gradient gel electrophoresis; FAME, fatty acid methyl ester analysis; GC, gas chromatography; PCR, polymerase chain reaction; PLFA, phospholipid fatty acid analysis; rep-PCR, repetitive sequence-based PCR; RISA, ribosomal intergenic spacer analysis; RT-PCR, PCR with reverse transcription; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.

the microflora, its diversity and function in soil has been reviewed elsewhere (Nannipieri et al., 2003). Approaches aiming at characterizing microbial communities in the environment are likely to go hand in hand with pure culture systems for detailed functional studies of biodegradative functions, to yield information about microbial enzymatic specificity and processivity (Galvão et al., 2005).

Table 1-4. Advantages and disadvantages of biochemical-based methods to study soil microbial diversity. Table modified from Kirk et al. (2004).

Method	Advantages	Disadvantages	Selected references
Plate counts	Fast, inexpensive	Unculturable microorganisms not detected, bias towards fast growing individuals, bias towards fungal species that produce large quantities of spores	Tabacchioni et al., 2000; Trevors, 1998
Community level physiological profiling (CLPP), sole carbon source utilization (SCSU)	Fast, highly reproducible, relatively inexpensive, differentiate between microbial communities, generates large amount of data, option of using bacterial, fungal plates or site specific carbon sources (Biolog)	Only represents culturable fraction of community, favours fast growing organisms, only represents those organisms capable of utilizing available carbon sources, potential metabolic diversity, not in situ diversity, sensitive to inoculum density	Classen et al., 2003; Garland, 1996; Garland & Mills, 1991
Fatty acid methyl ester analysis (FAME)	No culturing of microorganisms, direct extraction from soil, follow specific organisms or communities	If using fungal spores, a lot of material is needed, can be influenced by external factors, possibility that results can be confounded by other microorganisms	Graham et al., 1995: Siciliano & Germida, 1998; Zelles, 1999
Phospholipid fatty acid analysis (PLFA)	Changes in fingerprint can indicate change in community structure	No isolates obtained for further study	Kozdrój & van Elsas, 2001; van Hamme et al., 2003

Table 1-5. Characteristics of the direct and indirect DNA extraction methods used for microbial community DNA analysis. Table modified from Kozdrój & van Elsas (2001).

	DNA extraction method	
	Direct	**Indirect**
Cell origin	In situ lysis, microbial cells lysed directly in soil	Ex situ lysis, microbial cells lysed after separation from soil colloids
Cell lysis	Prevalence of mechanical lysis (e.g. bead-beating, sonication, freeze-thawing, cold disruption)	Chemical lysis using SDS, phenol, various detergents or enzymes (lysozyme, proteinase K or pronase)
DNA yield	Generally high (from total community)	Generally low (from microbial fraction separated)
Purity of DNA	Generally low (contaminated with humic acids, heavy metals, xenobiotics); require longer purification procedure	Generally high (contaminated with proteins, polysaccharides)
Representativeness for the community	High, but biased by extracellular DNA or plant cell DNA	Restricted to microorganisms extracted from soil colloids

1.3.1 Molecular biomonitoring methods to study the structural and functional diversity of bacteria and their catabolic genes

Molecular biomonitoring refers to the following up of biological phenomena at molecular level by using molecular biological, i.e. DNA/RNA-based, techniques without any genetic manipulation, such as introduction of marker genes. The use of molecular techniques to identify microorganisms (and their catabolic genes) is currently a key tool to study rhizosphere ecology (Pühler et al., 2004; Barea et al., 2005). Although giving valuable information on both culturable and unculturable microbia, the molecular biological methods have also their limitations. Biases appear at the stage of collecting DNA (Kirk et al., 2004; Kozdrój & van Elsas, 2001). First, the separation of nucleic acids from soil components might not be complete and can also cause shearing of DNA/RNA (direct extraction) (Table 1-5). In indirect extraction of nucleic acids, the detachment of different bacterial cells from soil particles and the lysis efficiency of different bacterial cells might vary. Second, the DNA/RNA extraction method and purification steps can influence the yield and intactness of nucleic acids. In addition, inhibitive compounds and contaminants can make the extraction troublesome. Widada et al. (2002) have reviewed the progress made in nucleic acid extraction and purification methods for environmental samples. Characteristics of the direct and indirect DNA extraction methods used for microbial community DNA analysis are presented in Table 1-5.

Microbial **diversity** consists of two elements, richness and evenness. The highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994; Kapur & Jain, 2004). **Structural** diversity of bacteria refers to taxonomic diversity. Special **functional** groups of bacteria could also be focused on. Most of the methods to study structural diversity can also be applied to study the genetic diversity of catabolic genes. More information about characterization of catabolic genes is presented in ch. 1.3.1.3. The amount (Q-PCR) and expression (RT-PCR) of catabolic genes reveals their functionality in soil.

1.3.1.1 Total community analysis – Bacterial diversity assessed by broad-scale molecular approaches

A holistic view of a microbial community in soil can be achieved by broad scale molecular approaches utilizing the DNA extracted from the total community within the soil (Kozdrój & van Elsas, 2001). These approaches include the reassociation kinetics of denatured DNA and the determination of the guanine plus cytosine (% G+C) content of DNA (Fig. 1-6; Table 1-6). The **rate of DNA reassociation** will depend on the similarity of sequences present (Torsvik et al., 1990a,b; Kirk et al., 2004). As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease. **Guanine plus cytosine content** (**% G+C**) determined by melting or density gradient centrifugation is based on the knowledge that microorganisms differ in their G+C content and that taxonomically related groups only differ between 3% and 5% (Holben & Harris, 1995; Tiedje et al., 1999; Kirk et al., 2004).

1.3.1.2 Partial community analysis – Identification of community members and gene diversity by narrow-scale molecular approaches

1.3.1.2.1 PCR-based genetic fingerprinting techniques

Soil microbial communities can be genetically fingerprinted and the community members identified by comparison with fragment sizes or sequences in databases (Fig. 1-6). In genetic fingerprinting, DNA is extracted from the environmental sample and purified. Target DNA is amplified using universal or specific primers and the resulting products are separated in different ways (Kirk et al., 2004) (Table 1-6). Typically, 16S rRNA region (also 23S; 18S and internal transcribed spacer, ITS, for fungi) is targeted by primers for diversity studies (Kirk et al., 2004; Lynch et al., 2004). Thus, DNA fingerprinting techniques have been used to map the phylogenetic

distribution of complex communities based on rRNA gene sequences, but their application for mapping CATABOLIC GENE sequence diversity is at an early stage (Galvão et al., 2005).

The PCR-based community fingerprinting techniques have several ADVANTAGES (Wintzingerode et al., 1997). They are 1) rapid, and allow parallel analyses of multiple samples, 2) reliable and highly reproducible, 3) provide both qualitative and quantitative information on populations within a community, and 4) allow the assessment of phylogenetic affiliation of community members by comparison with fragment sizes or sequences in databases. However, PCR-based techniques also present several DRAWBACKS such as 1) bias in PCR amplification due to preferential amplification of target DNAs from some bacteria (influenced by G+C content, primer specificity, different copy number of target genes), 2) formation of chimeric molecules, 3) derivation of several different PCR amplicons from a single bacterial strain due to the presence of several operons, and 4) the numbers of amplicons from complex communities can be too high to be readily separated and resolved (Suzuki & Giovannoni, 1996; Wintzingerode et al., 1997; Kirk et al., 2004; Kozdrój & van Elsas, 2001).

Genetic fingerprinting methods are limited in their ability to discriminate between communities with high diversity where the number of PCR amplicons are too high to be readily resolved (Lynch et al., 2004). In COMPLEX COMMUNITIES, rRNA-based fingerprinting techniques can be used to partially analyze the community, focusing on a SUBSET of the community by applying primers targeting specific phylogenetic (e.g. Archaea) or functional (e.g. methanogenic) groups of microorganisms (Lynch et al., 2004). Another approach to identify community members is to apply specific ENRICHMENTS to enhance the growth of the microorganism of interest (Lynch et al., 2004). This strategy is particularly useful in studies of functional groups or guilds.

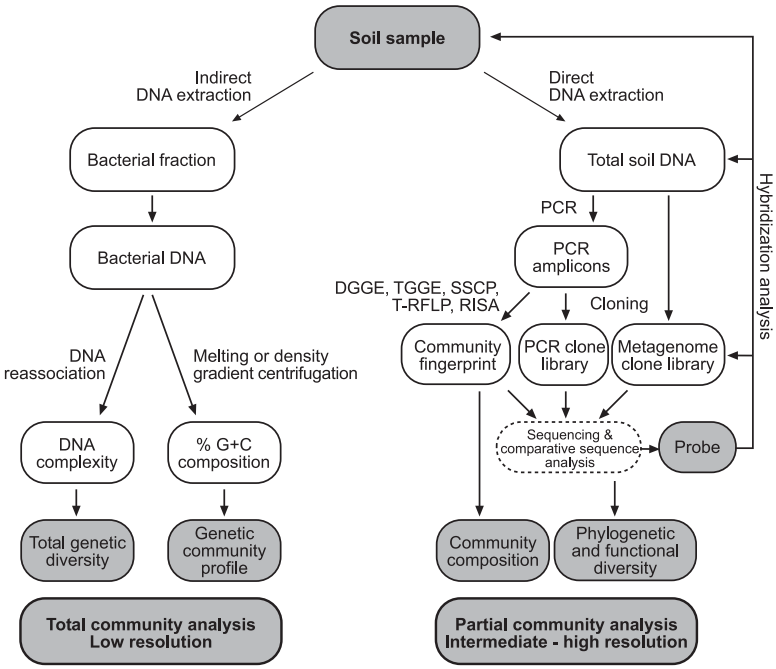


Fig. 1-6. Culture-independent molecular methods for the analyses of microbial communities in soil. Figure adapted from Lynch et al. (2004). DGGE, denaturant gradient gel electrophoresis; PCR, polymerase chain reaction; RISA, ribosomal intergenic spacer analysis; SSCP, single strand conformation polymorphism; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism; % G+C, mole % guanine and cytosine.

The PRINCIPLES of different fingerprinting techniques are described in the following text while the potentials, limitations and applications of them are compared in Table 1-6.

In **denaturant gradient gel electrophoresis** (**DGGE**) and **temperature gradient gel electrophoresis** (**TGGE**) analysis, DNA fragments with the same length but different nucleotide sequences are separated (Muyzer et al., 1993; Heuer & Smalla, 1997). Separation is based on differences in migration of the molecules with different sequences that thus have a different melting behaviour in polyacrylamide gel containing a linear gradient of denaturants or a linear temperature gradient (Kozdrój & van Elsas, 2001). DGGE/TGGE banding patterns have been analyzed for diversity studies based on the number and intensity of the DNA bands as well as similarity between treatments (Kirk et al., 2004). Specific DGGE/TGGE bands can also be excised from gels, re-amplified and sequenced or transferred to membranes and hybridized with specific primers to provide more structural or functional diversity information (Theron & Cloete, 2000; Kirk et al., 2004). Information about the taxon composition of the community can be obtained with phylogenetic probes (Lynch et al., 2004). Holben et al. (2004) introduced GC fractionation combined with DGGE (GC-DGGE) to enhance assessment of microbial community diversity and detection of minority populations of microbes. The use of catabolic genes as target in DGGE/TGGE analysis would provide information on the diversity of specific groups of microorganisms competent in a defined function such as pollutant degradation or methanotrophy (Fjellbirkeland et al., 2001; Knief et al., 2003; Kirk et al., 2004). The occurrence of genes coding for desulfuration of hydrocarbons (*dsz*) in a polluted field soil has been shown by DGGE, while these genes were not detected in unpolluted soils (Duarte et al., 2001). In addition, DGGE/TGGE analysis of PCR amplicons derived from rRNA molecules by RT-PCR might give fingerprints of the metabolically active microbial populations (Heuer & Smalla, 1997; Lynch et al., 2004).

Single strand conformation polymorphism (**SSCP**), like DGGE/TGGE, detects sequence variations between different PCR amplicons normally derived from variable regions of the rDNA (Lee et al., 1996; Stach et al., 2001; Lynch et al., 2004). In SSCP one primer is phosphorylated at the 5' end, and the phosphorylated strand of the PCR amplicons is selectively digested with lambda exonuclease. The intact strands are separated by electrophoresis under nondenaturing conditions (low temperature) in a polyacrylamide gel optimal for SSCP. This optimal gel restricts duplex formation but allows intra-molecular folding of the DNA strands. The method is based on the differential intra-molecular folding of single-stranded DNA that is itself dependent upon DNA sequence variations. Thus, DNA secondary structure alters the electrophoretic mobility of the single-stranded PCR amplicons enabling them to be resolved.

Terminal restriction fragment length polymorphism (**T-RFLP**) analysis is based on the restriction endonuclease digestion of fluorescent end-labelled PCR amplicons (Avaniss-Aghajani et al., 1994; Liu et al., 1997; Osborn et al., 2000; Lynch et al., 2004). T-RFLP measures only the terminal restriction fragment of each 16S rRNA gene, and thereby the complexity of the RFLP pattern is reduced and every visible band is representative of a single ribotype or operational taxonomic unit (OTU) (Liu et al., 1997; Kozdrój & van Elsas, 2001). In addition to analyses based on housekeeping genes (e.g. rDNA), T-RFLP has been used to analyze enzyme-coding (functional) genes such as mercury resistance genes, and particulate methane monooxygenase genes (Bruce, 1997; Horz et al., 2001; Lynch et al., 2004).

Restriction fragment length polymorphism (**RFLP**), also known as **amplified ribosomal DNA restriction analysis** (**ARDRA**) is a DNA fingerprinting technique based on restriction enzyme digestions and agarose gel electrophoresis of PCR-amplified 16S rRNA genes using primers for conserved regions (Tiedje et al., 1999; Kozdrój & van Elsas, 2001). It is a powerful tool for bacterial identification and classification at species level (Massol-Deya et al., 1995) and it has been used to group and classify large sets of isolates and clones for instance in Cu contaminated soil (Smit et al., 1997).

Ribosomal intergenic spacer analysis (**RISA**) or **automated ribosomal intergenic spacer analysis** (**ARISA**) is based on the length and/or the sequence polymorphism of the ribosomal intergenic spacer (IGS) region between the 16S and 23S rRNA genes (Borneman & Triplett, 1997; Fisher & Triplett, 1999; Ranjard et al., 2000b; Kirk et al., 2004; Lynch et al., 2004). PCR

Table 1-6. Molecular methods for soil microbial diversity studies. Data collected mainly from Kirk et al. (2004) and Lynch et al. (2004) but also from Kozdrój & van Elsas (2001) and Widada et al. (2002).

Method	Type of information	Resolution	Advantages	Disadvantages	Application in soil microbial analysis	Selected references
Total community analysis						
DNA reassociation rate	Total genetic diversity, theoretical "species" number, community "genome size"	Low	Total DNA extracted, not influenced by PCR biases	Lack of sensitivity, sequences need to be in high copy number to be detected, dependent on lysing and extraction efficiency	Global analysis of the genetic potential of communities, comparative analysis of the overall biodiversity	Torsvik et al., 1990a,b,1996; Sandaa et al., 1999b
Mole % G+C composition	Genetic community profile, overall community composition	Low	Not influeced by PCR biases, includes all DNA extracted, quantitative, includes rare members of community	Requires large quantitities of DNA, dependent on lysing and extraction efficiency	Comparative analysis of the overall changes in community composition	Holben & Harris, 1995; Nüsslein & Tiedje, 1998, 1999; Tiedje et al., 1999; Øvreås et al., 1998
Partial community analysis						
PCR-based genetic fingerprinting techniques						
PCR-DGGE/TGGE - sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members	Inter-mediate	Large number of samples can be analyzed simultaneously, reliable, reproducible, rapid	PCR biases, dependent on lysing and extraction efficiency, sample handling (i.e. storage time before extration) can influence community, one band can represent more than one species (co-migration) or vice versa, only detects dominant species	Comparative analysis of community structure, spatial and temporal changes in community composition; phylogenetic and functional diversity	Muyzer et al., 1993; Muyzer 1999; Duinevald et al., 2001; Niemi et al., 2001; Smalla et al., 2001; Heuer & Smalla, 1997; Brim et al., 1999; Fjellbirkeland et al., 2001; Knief et al., 2003; Kitagawa et al., 2001
PCR-SSCP - sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members	Inter-mediate	Same as DGGE/TGGE, but easier to carry out: no GC clamp, no gradient	Same as DGGE/TGGE, PCR biases, some ssDNA can form more than one stable conformation	Comparative analysis of community structure, spatial and temporal changes in community composition; phylogenetic and functional diversity	Orita et al., 1989; Lee et al., 1996; Tiedje et al., 1999; Schwieger & Tebbe, 1998; Stach et al., 2001; Junca & Pieper, 2003
PCR-T-RFLP - T-RF size distribution	Community composition, relative abundance of numerically dominant community members	Inter-mediate	Simpler banding patterns than in RFLP, can be automated, large number of samples, highly reproducible, compare differences in microbial communities	Dependent on extraction and lysing efficiency, PCR biases, type of Taq can increase variability, choice of universal primers, choice of restriction enzymes will influence community fingerprint	Comparative analysis of distribution of microbial populations, monitoring changes in community composition; phylogenetic and functional diversity	Liu et al., 1997; Tiedje et al., 1999; Dunbar et al., 2000; Osborn et al., 2000; Avaniss-Aghajani et al., 1994 Bruce, 1997; Horz et al., 2001
PCR-ARDRA, PCR-RFLP - RF size distribution	Genetic fingerprinting of simple communities, populations or phylogenetic groups, discrimination at species and higher taxonomic levels	High	Detect structural changes in microbial community	PCR biases, banding patterns often too complex	Comparative analysis of microbial population dynamics, diversity within phylogenetic or functional groups of microorganisms	Liu et al., 1997; Tiedje et al., 1999; Massol-Deya et al., 1995; Smit et al., 1997; Fries et al., 1997

Table 1-6 *(continued)*

PCR-(A)RISA - IGS size distribution	Genetic fingerprinting of populations or phylogenetic groups, simultaneously analysis of different microbial groups, discrimination at species (strain) or group level	High	Highly reproducible community profiles, can be automated (ARISA)	Requires large quantities of DNA, resolution tends to be low, PCR biases	Comparative analysis of microbial population dynamics, diversity within phylogenetic or functional groups of microorganisms	Fisher & Triplett, 1999; Borneman & Triplett, 1997; Ranjard et al., 2000a,b, 2001
rep-PCR - size distribution between repetetive sequences	Genomic fingerprinting of chromosome structure, identification and classification of strains at species and subspecies level	High	May be useful to develop probes to detect community changes caused by an environmental change	Limited usage with complex communities, the sequence of the repeated region needs to be known, PCR biases for primers	Monitoring specific population in microbial communities, assessing the diversity of bacterial isolates and cloned genes	Versalovic et al., 1991, 1994; de Bruijn, 1992; Tiedje et al., 1999; Johnsen et al., 2001; Roane & Pepper, 2000
<u>Cloning techniques</u>						
PCR of rDNA - cloning and sequencing	Phylogenetic diversity, identification of community members	High	Sequencing is routine, high sensitivity	PCR biases, sequencing of thousands of clones is cumbersome	Phylogenetic diversity of community members	Tiedje et al., 1999; Borneman et al., 1996; Raskin et al., 1994; Muyzer & Smalla, 1999
PCR/RT-PCR of catabolic genes - cloning and sequencing	Functional diversity, identification of catabolic genes, gene expression	High	Sequencing is routine, high sensitivity	PCR biases, sequencing of thousands of clones is cumbersome	Comparative analysis of the functional potential of communities	Yeates et al., 2000; Wilson et al., 1999 Widada et al., 2002
<u>Hybridization techniques</u>						
RNA dot/slot blot hybridization	Phylogenetic identification of metabolic active community members	Inter-mediate	Total DNA extracted, not influenced by PCR biases, study DNA or RNA	Lack of sensitivity, sequences need to be in high copy number to be detected, dependent on lysing and extraction efficiency	Qualitative and quantitative analysis of metabolic active populations in communities, phylogenetic information on active community members	Cho & Tiedje, 2001; Guo et al., 1997; Head et al., 1998; Theron & Cloete, 2000
FISH	Detection and specific counting of metabolic active microorganisms	Inter-mediate	Spatial distribution can be visualized at cellular level *in situ*	Lack of sensitivity (TSA-FISH technique can improve), laborious	Comparative analysis of community structure, detection and identification of active cells, direct phylogenetic information on community members	Amann et al., 1990, 1995; Hahn et al., 1992; Schramm et al., 1996; Johnsen et al., 2001; Wagner et al., 2003; Galvão et al., 2005
DNA macro- and microarrays	Phylogenetic or functional diversity of microbial communities	Inter-mediate	Same as na hybridization, thousands of genes can be analyzed, if using genes or DNA fragments, increased specificity	Only detect most abundant species, only accurate in low diversity systems	Assessing influence of pollutants on gene expression, analysis of microbial community composition of the most dominant culturable species (RSGP)	Hubert et al., 1999; Cho & Tiedje, 2001; Greene & Voordouw, 2003; Galvão et al., 2005; Rhee et al., 2004
Catabolic gene probing	Functional diversity, identification of catabolic genes	Inter-mediate	Detect genes with function of interest, mRNA detection can reveal information about expression	Limited to known genes, activity cannot be inferred from presence of genes alone	Comparative analysis of community composition, abundance, and expression of recalcitrant-degrading bacteria	van Hamme et al., 2003; Hamann et al., 1999; Guo et al., 1997; Stapleton et al., 1998

Methods: ARDRA, amplified ribosomal DNA restriction analysis; ARISA, automated ribosomal intergenic spacer analysis; DGGE, denaturant gradient gel electrophoresis; FISH, fluorescence *in situ* hybridisation; PCR, polymerase chain reaction; rep-PCR, repetitive sequence-based PCR; RFLP, restriction fragment length polymorphism; RISA, ribosomal intergenic spacer analysis; RSGP, reverse sample genome probing; RT-PCR, PCR with reverse transcription; SSCP, single strand conformation polymorphism; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism; TSA-FISH, tyramide signal amplification FISH; % G+C, mole % guanine and cytosine; Others: IGS, ribosomal intergenic spacer region; na, nucleic acids (DNA, RNA); RF, restriction fragment; T-RF, terminal restriction fragment.

products are either separated by agarose gel electrophoresis, or denaturated and separated on a polyacrylamide gel under denaturing conditions (Kirk et al., 2004). In RISA and ARISA, respectively, silver stain and fluorescence (higher sensitivity) is used for detection (Kirk et al., 2004). Several primers targeting different phylogenetic groups in the same sample can be used to evaluate simultaneously the population dynamics of different microbial phylotypes within a community (Ranjard et al., 2000a,b; Lynch et al., 2004).

Differences in GENOMIC FINGERPRINTS of chromosome structure among isolated bacterial stains can be obtained with highly repeated sequence characterization (Tiedje et al., 1999; Kozdrój & van Elsas, 2001; Kirk et al., 2004). In **repetitive sequence-based PCR** (**rep-PCR**) method specific primers, such as ERIC, REP, BOX and $(GTG)_5$, bind to randomly interspersed repetitive DNA sequences existing throughout the genome (Versalovic et al., 1991, 1994; de Bruijn 1992). The amplified fragments are separated in agarose gel electrophoresis. Because the rep-PCR is most effectively used when it is targeted to populations selected to be of a certain phenotypic or taxonomic group, mixed natural communities of bacteria in contaminated soils have not been studied by this method (Kozdrój & van Elsas, 2001). However, Roane & Pepper (2000) used ERIC-PCR to genetically distinguish the bacterial isolates from soils contaminated with different levels of Cd. The isolates were identified, using 16S rRNA gene sequencing, as *Arthrobacter*, *Bacillus*, and *Pseudomonas* spp. that differed in their resistance to Cd.

1.3.1.2.2 Cloning techniques

Cloning techniques produce clone libraries (Fig. 1-6; Table 1-6). The cloned amplicons can be compared by fingerprinting methods such as ARDRA (Sandaa et al., 2001). rRNA gene clones can also be classified by dot/slot blot hybridization with phylogenetic probes (Manz et al., 1992). Sequencing of the cloned genes and comparing the sequences with those obtained from databases provides information about affiliation of the cloned sequences (Sandaa et al., 1999a,b). More information about cloning techniques is presented in Table 1-6 and Fig. 1-6.

1.3.1.2.3 Hybridization techniques

By choosing sequences in conserved, variable and hypervariable regions of the rRNA, probes can target phylogenetic groups at different taxonomic levels, ranging from domain to subspecies (Lynch et al., 2004). Domain- to species-specific oligonucleotide or polynucleotide probes are usually tagged with fluorescent markers (derivatives of fluorescein and rhodamine) at the 5'-end (Kirk et al., 2004). Hybridization methods can help to resolve the species composition within specific parts (organism groups) of the community (Lynch et al., 2004). **Slot/dot blot and Southern blot hybridization** of community fingerprints (e.g. DGGE profiles) with phylogenetic probes has proved particularly useful in studying changes in communities and in identifying the numerically dominant community members (Øvreås et al., 1997). A combination of slot blot hybridization and **fluorescence *in situ* hybridization** (**FISH**) was used to distinguish the community structure of low and high metal-contaminated soils (Chatzinotas et al., 1998; Sandaa et al., 1999b). More information about hybridization techniques is presented in Table 1-6 and Fig. 1-6.

DNA macro- and microarrays have been used widely for studying gene expression of bacteria, but the application of this tool in environmental microbiology is still being optimized (Galvão et al., 2005). Three types of arrays have been described for looking at environmental DNA that differ on the kind of DNA arrayed: 1) functional gene arrays (FGAs), 2) community genome arrays (CGAs), and 3) phylogenetic genome arrays (PGAs) (Galvão et al., 2005). CGAs (made with genomic DNA isolated form environmental samples or pure cultures) and PGAs (made with oligonucleotides from rRNA genes) give indirect information on the biodegradation gene landscape of an environmental sample. FGAs contain probes for genes encoding enzymes involved in biodegradation and biotransformation, such that by design they probe the functional (as defined by gene) diversity in microbial communities, as well as enabling the quantification

of activity by detecting mRNA. FGAs thus enable the diversity to be probed of gene sequences implicated in pollutant degradation. Rhee et al. (2004) have detected genes involved in biodegradation and biotransformation in microbial communities by using microarrays.

1.3.1.3 Biodegradation gene pool – finding variants and novelties

By focusing on the biodegradation gene pool, this chapter gives an overview of available methods to CHARACTERIZE catabolic genes QUALITATIVELY (diversity) and QUANTITATIVELY (amount, expression), in addition to finding NEW enzymatic ACTIVITIES.

PCR can be used for sensitive detection of specific catabolic genes in environmental samples. PCR products can then be fingerprinted (ch. 1.3.1.2) or cloned and sequenced to find out sequence VARIANTS (GENE DIVERSITY). The SENSITIVITY of PCR can be ENHANCED by combining PCR with DNA probes, by running two rounds of amplification using nested primers or by using real-time detection systems (Widada et al., 2002). Three techniques have been developed for estimating the AMOUNT of DNA by PCR (**quantitative PCR, Q-PCR**) reviewed by Widada et al. (2002): most probable number PCR (MPN-PCR), replicative limiting dilution PCR (RLD-PCR), and competitive PCR (cPCR). RT-cPCR, i.e. **quantitative reverse transcription PCR** (**Q-RT-PCR**), can be used to monitor catabolic ACTIVITY of cells. Fey et al. (2004) established Q-PCR and Q-RT-PCR methods for determining the presence and metabolic activity of pathogenic bacteria in environmental samples. Baldwin et al. (2003) have enumerated aromatic oxygenase genes by multiplex and real-time Q-PCR. Q-PCR to monitor degradation genes such as *bphC*, *xylE*, *nahA* and *ndoB* are available (Ducrocq et al., 1999; Milcic-Terzic et al., 2001).

Galvão et al. (2005) have summarized well the **SEQUENCE-DEPENDENT** and **SEQUENCE-INDEPENDENT APPROACHES** (Fig. 1-7) to identify VARIANTS of genes or new CANDIDATE GENES, respectively, in pathways for biodegradation of recalcitrant and xenobiotic molecules and thus, the enzymatic diversity of microbial consortia in polluted sites: "Most current procedures to identify genes for given catalysts in microbial communities are BASED ON SEQUENCE similarity to known enzymes. In these cases, some information on the amino acid sequence of the pursued protein or on the corresponding DNA is needed before setting out on the search for new VARIANTS. Applicable techniques in this context include **fluorescence *in situ* hybridization** (**FISH**) of radioactive or fluorescent oligonucleotide probes, straight **PCR** of environmental DNA or **RT-PCR** of mRNA for amplifying target sequences and monitoring their levels of presence or expression, and ***in situ* RT-PCR** to the same end. The use of DNA-dependent techniques, such as those that are PCR or oligonucleotide based, enables environmental monitoring simultaneously of the presence and activity of genes for biodegradative enzymes. A second class of approaches

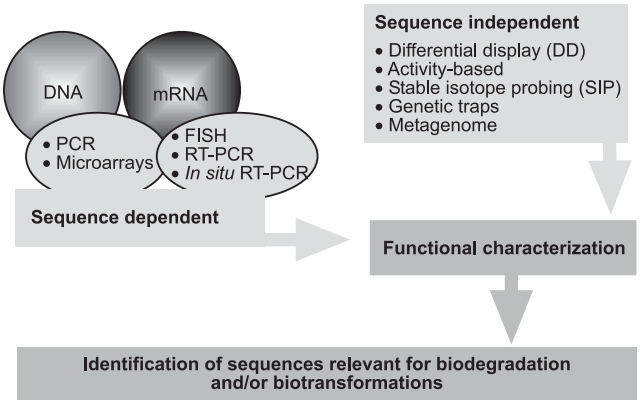


Fig. 1-7. General approaches for exploring the environmental biodegradation and/or biotransformation gene pool. Scheme modified from Galvão et al. (2005). See text 1.3.1.3 for details.

attempts to identify environmental DNA segments that are CANDIDATES to encode biocatalysts in a SEQUENCE-INDEPEDENT fashion. These include cloning of genes expressed only in conditions of induction (**differential display, DD**), direct detection of **new reactions** (optimally associated to a color change in the substrate), density enrichment of the genomes of those members of the community that metabolize a certain substrate labeled with ^{13}C (**stable isotope probing, SIP**) and **genetic traps** to translate the desired enzymatic reaction into a selectable or scorable property of the surrogate host of environmental DNA."

Metagenome analysis is also SEQUENCE-INDEPENDENT and involves cloning DNA directly from soil (Paul et al., 2005). Metagenome is the total genomic DNA derived from microbial communities (Lynch et al., 2004) and metagenomics is the genomics of total microbial DNA extracted directly from environments (Paul et al., 2005). In metagenome analysis, large genomic fragments are cloned into bacterial artificial chromosomes (BAC), fosmids and/or cosmid vectors such that the whole metagenome is represented by a finite number of screenable clones (Paul et al., 2005). Efficient BAC shuttle vectors are now available for the construction of environmental libraries in hosts such as *E. coli*, *Pseudomonas* and gram-positive *Streptomyces* (Paul et al., 2005). Metagenomics has been successfully used to access the biosynthetic diversity of microorganisms from varied environments (reviewed by Paul et al., 2005). Metagenomic profiling promises enormous potential for identifying NOVEL ENZYMES or pathways involved in the biodegradation of poorly and inefficiently degraded pollutants.

1.3.2 Microbial communities and their catabolic genes as ecological indicators of contamination

Nowadays there is a wide range of methods available to study microbial and catabolic gene diversity in pristine and contaminated soils. Each method has its limitations and only provides a partial picture of one aspect of soil microbial diversity. With our current knowledge, it is impossible to evaluate the effectiveness of each method (Kirk et al., 2004). Thus, the best way to study soil microbial diversity would be to use a VARIETY OF METHODS with different endpoints and degrees of resolution to obtain the broadest picture possible and the most information regarding the complex microbial community (Kirk et al., 2004; Kozdrój & van Elsas, 2001).

In addition to the data presented in chapters 1.2.3-1.2.4, Lynch et al. (2004) and Kirk et al. (2004) have also reviewed the research assessing the impact of pollution and agricultural management on microbial diversity and community structure in soil using especially molecular methods with different levels of resolution or using both traditional and molecular methods. The results of these studies suggest that quantitative measures of microbial diversity and qualitative analysis of community structure can discriminate between soil samples subjected to different levels of pollution and be useful **ECOLOGICAL INDICATORS** of stress and perturbation, which are often seen as reduced diversity (Lynch et al., 2004).

It is generally thought that a diverse populations of organisms will be more resilient to stress and more capable of adapting to environmental changes (Kirk et al., 2004). It is not known what reductions in diversity mean to ecosystem functioning and it is important for sustainability of ecosystems that the LINK BETWEEN DIVERSITY AND FUNCTION be examined and better understood (Kirk et al., 2004). Lynch et al. (2004) suggested that DNA and mRNA measurements should be combined with the application of the proteomic approach to soil so as to have measurements of protein expression in soil. This combined approach might give better insights into the links between microbial diversity and soil functionality.

2 AIMS OF THE STUDY

2.1 Research idea

There are thousands of oil-contaminated sites, e.g. old petrol stations, in need to be cleaned in Finland. The success of inoculating soil directly with bacterial degradaders most efficient in laboratory conditions has been a game of chance for bioremediation in nature. Instead, indigenous bacteria already adapted to living in soil with plant roots and showing good competitive growth could lead the way also to effective rhizoremediation. In this work, an agriculturally well-known interaction between N_2-fixing leguminous plant and bacteria was applied in a new context, bioremediation of oil-contaminated soils. Our model system was a perennial forage legume, goat's rue, *Galega orientalis* (Fig. 2-1), and its microsymbiont *Rhizobium galegae* bv. *orientalis* with other rhizosphere bacteria. In addition to evaluate the intrinsic biodegradation potential of *R. galegae* and other rhizosphere bacteria, we also tested the effect of a bioaugmentation bacterium *Pseudomonas putida* PaW85/pWW0, which is able to degrade BTEX compounds effectively based on the archetypal TOL plasmid pWW0.

Nitrogen fixing legume provides a supply of nitrogen in addition to other nutrients such as carbon sources to rhizosphere bacteria, while some of these bacteria could protect the plant from toxic effects by degrading oil compounds (Fig. 2-2). Therefore, rhizoremediation could work in such a way that the legume stimulates bacterial proliferation and *m*-toluate (a model compound of BTEX) degradation, which, in turn, might facilitate roots to grow deeper into the contaminated soil. BTEX biodegradation would also be accelerated based on the spread of rhizobacteria with roots and the capability of TOL plasmids to naturally spread in oil-contaminated legume rhizosphere, where plant roots offer a solid surface for conjugation. At the same time, plant roots would physically improve the soil structure and aeration.

Fig. 2-1. Goat's rue (*Galega orientalis*).

The rhizoremediation of oil-contamianted soil based on the legume's rhizosphere bacteria would be a new, ecological, economical and aesthetical bioremediation method. The method could easily be applied to sites with large area with fairly low level of contaminants. It was evident that the research, which would lead to a ready bioremediation application *in situ*, would be too ambitious for a single dissertation thesis. In addition, there was no possibility for complete chemical analyses during this work to confirm the removal of contaminants and thus, the success of our bioremediation method. Therefore, the focus of this first thesis in our bioremediation project was primarily on developing and subsequently applying molecular biological methods for biomonitoring of rhizoremediation to get more information on the biological aspects behind rhizoremediation.

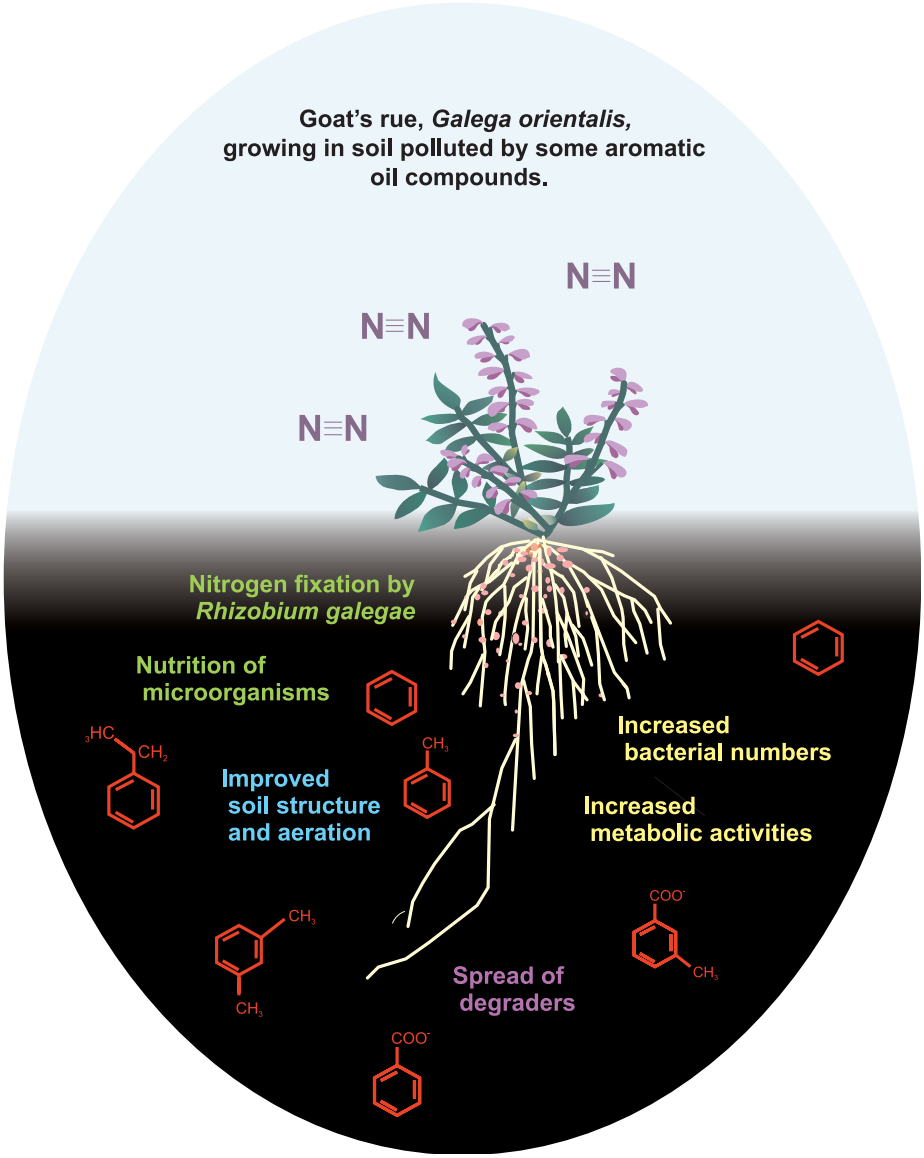


Fig. 2-2. Research idea about bioremediation in *Galega* rhizosphere.

2.2 The main and specific aims

The main aims of the thesis were

1. To develop molecular methods for biomonitoring (III, V) and
2. To investigate biological phenomena during rhizoremediation of oil-contaminated soil
- by studying visible plant responses (I, III, IV) and
- by applying molecular biomonitoring methods for bacteria and their catabolic genes (III, IV, V).

The specific aims of the research were

1. To evaluate *in vitro* and in greenhouse the potential use of the symbiotic *Galega orientalis – Rhizobium galegae* interaction alone and in association with *P. putida* PaW85 for the rhizoremediation of oil-contaminated soils (I).

2. To develop molecular toolbox for grouping of heterogeneous rhizosphere bacteria (III) and to design gene-specific primers for TOL plasmid detection (V). In addition, the need for a method to detect other kinds of aromatics-degrading plasmids was obvious (V).

3. To study genetic diversity of culturable, *m*-toluate tolerating rhizobacteria by the developed taxonomical characterization and identification methods (III, IV).
- to isolate indigenous *m*-toluate tolerating bacteria from oil-contaminated rhizosphere of *G. orientalis* and thus, to establish a culture collection of rhizobacteria

4. To characterize the isolated rhizobacteria in relation to the degradation of oil compounds:
- to phenotypically characterize the ability of the rhizobacteria to degrade *m*-toluate (III, IV) and
- to detect and profile aromatics-degrading plasmids, specifically TOL plasmids, from rhizobacteria (V).

5. To explore the potential rhizosphere effect (bacterial abundance and community composition) of *Galega orientalis* in oil-contaminated soil in the field experiment (IV).

6. To monitor the horizontal transfer of TOL plasmids (V):
- to conjugate a TOL plasmid pWW0 from the genus *Pseudomonas* to the genus *Rhizobium* in order to combine BTEX degradation and N_2 fixation genes to the same bacterium and
- to indicate horizontal TOL plasmid transfer between rhizobacteria and thus, the gene dynamics during rhizoremediation.

7. To monitor changes in the diversity of TOL plasmids and bacteria in rhizosphere communities through direct DNA analysis (V).

3 MATERIALS AND METHODS

3.1 Bacterial strains, cultivation and maintenance

Pseudomonas strains and *E. coli* were grown on tryptone yeast extract agar (TY) (Beringer, 1974) or on R2A Medium (LAB M™) at +28ºC for 1 d. *P. oleovorans* was grown on ATCC medium 910 supplemented with 1% *n*-octane for 7 d. *Rhizobium* and *Agrobacterium* strains were grown on yeast extract mannitol agar (YEM) supplemented with Congo red at +28ºC for 2 d (Lindström et al., 1983). Antibiotics and/or *m*-toluate were added for strain and/or TOL plasmid selection, when appropriate.

A total of 50 *m*-toluate tolerating bacterial strains were isolated from a greenhouse rhizoremediation experiment from the oil-contaminated rhizosphere of *Galega orientalis* (Paper III). These strains and their relevant characteristics are listed in Table 1 in Paper III. The strains were grown and isolated on DEF8-based agar (Lindström & Lehtomäki, 1988), supplemented with cycloheximide (CHX) to inhibit fungal growth, at +28°C for 2-3 d. Glucose, mannitol or rhizosphere extract were used alone or in different combinations as carbon source. For some isolations soil extract agar (SEA) containing glucose was used. The selective agent in all the media was *m*-toluate, which was used in various concentrations.

A total of 208 bacterial strains were isolated from an oil-contaminated field experiment with *Galega* rhizosphere (Paper IV). These strains were grown on *m*-toluate containing TY plates incubated at +15°C for 7 d. In addition, DEF8-based minimal and rich media amended with root and rhizosphere extract were in use. In contrast to the minimal DEF8, rich DEF8 contained also glucose, mannitol and DEF-vitamin solution.

The bacterial reference strains used for aromatic hydrocarbon degradation are listed in Table 1 in Paper V. The characteristics of their degradation plasmids and genes are presented in Table 5a in Paper V. Eight of the *m*-toluate tolerating strains isolated in Paper III were able to degrade *m*-toluate. The identification of their extradiol aromatic ring-cleavage dioxygenase (EDO) harboring degradation plasmids is presented in Table 5b in Paper V.

Bacterial strains were maintained on agar plates at +4°C. The strains were recultured on fresh agar plates approximately every month. The strains were stored in corresponding culture broth with 15% (v/v) glycerol at -70°C. For long time storage the strains were freeze-dried and deposited in HAMBI, the Microbial Culture Collection at the Department of Applied Chemistry and Microbiology, University of Helsinki, Finland.

3.2 Preparation of rhizosphere, soil and root extracts

Rhizosphere and soil extracts were prepared as described in Paper III. Root extract was prepared according to Zaat et al. (1988) with the modifications made by Suominen et al. (2003) as follows. Seeds of *Galega orientalis* were surface-sterilized by shaking in 70% ethanol for 30 s, three times in sterile water for 10 min, once in 0.1% $HgCl_2$ solution for 5 min and six times in sterile water for 10 min (Lipsanen & Lindström, 1988; Räsänen et al., 1991). Sterilized seeds were aseptically transferred onto yeast extract mannitol agar plates (YEM) supplemented with Congo red (R) (Merck; 25 mg/ml) (Lindström et al., 1985) and germinated in the dark at room temperature for 2-3 d until the roots were about 1 cm long. Seedlings (3 per tube) were grown on a steel net placed 3-5 mm above sterile 0.5 x Jensen medium (20 ml) (Vincent, 1970) in plant tubes (∅ 2 cm x 20 cm) so that the roots reached the Jensen solution. The shoots were grown in a growth chamber (Sanyo Growth Cabinet) at +20°C with an 18-h light and 6-h dark period for 4-7 d until the roots were 4-5 cm long. The shoots, net and seed shells were aseptically removed from the tubes and the sterility of the root extract was checked by incubating an aliquot on YEMR plates at +28°C for at least 4 d. All the extracts were stored at -20°C.

3.3 Experimental design of the greenhouse and the field lysimeter experiments

A nitrogen-fixing leguminous plant, goat's rue (*Galega orientalis*), and its microsymbiont *Rhizobium galegae* bv. *orientalis* as well as intrinsic rhizosphere bacteria were used as the model system for the bioremediation studies in this thesis. *Pseudomonas putida* PaW85 harboring the archetypal TOL plasmid pWW0 was added as a bioaugmentation bacterium to enhance the degradation of oil (BTEX) compounds.

The greenhouse experiment was done in 9-l pots with 20 seeds of *G. orientalis*. Plants were grown in three different ways: no inoculation, seed-inoculation with *R. galegae,* or rhizobia-inoculated seeds with *P. putida* bioaugmentation in peat layer. Soils used were oil-contaminated soil with 3% THC, *m*-toluate-polluted (2000 mg/l) agricultural soil, and uncontaminated agricultural soil.

The experimental design of the field lysimeter experiments is shown in Fig. 1 in Paper IV. This industrial oil-contaminated soil contained 200 mg/kg mineral oils. In the three-month field lysimeter experiment also *m*-toluate (2000 mg/l) was added into this oil-contaminated soil (Paper V).

3.4 Rhizosphere samples for direct DNA analysis

The analyses conducted directly at DNA level were performed with rhizosphere samples from a three-month field lysimeter experiment (Paper V).

3.5 PCR primers

PCR primers and conditions used in this study are summarized in Table 3-1.

3.6 Methods

The methods used for specific purposes in this study are described in detail and thus, in easy-to-repeat format in the original publications I and III-V, and they are summarized in Table 3-2.

3.7 Analysis of biological data

Computer programs used for bioinformatics in this study are summarized in Table 3-3.

Table 3-1. PCR primers and conditions used in the thesis.

Primer[a]	Sequence[b]	Position in reference species[c]	Product size (bp)	PCR conditions[d]	Paper	Reference
rep-PCR						
REP1R-I	5'-III ICG ICG ICA TCI GGC-3'			95° (94°, 40°, 65°) 65° (30)	III	Versalovic et al., 1991
REP2-I	5'-ICG ICT TAT CIG GCC TAC-3'			6' (1', 1', 8') 16'		
ERIC1R	5'-ATG TAA GCT CCT GGG GAT TCA C-3'			95° (94°, 52°, 65°) 65° (30)	III	Versalovic et al., 1991
ERIC2	5'-AAG TAA GTG ACT GGG GTG AGC G-3'			7' (1', 1', 8') 16'		
BOXAIR	5'-CTA CGG CAA GGC GAC GCT GAC G-3'			95° (94°, 52°, 65°) 65° (30)	III	Versalovic et al., 1994
				7' (1', 1', 8') 16'		
$(GTG)_5$	5'-GTG GTG GTG GTG GTG-3'			95° (94°, 52°, 65°) 65° (30)	III,	Versalovic et al., 1994
				7' (1', 1', 8') 16'	IV	
16S rRNA gene PCR-RFLP						
fD1 (pA)	5'-AGA GTT TGA TCC TGG CTC AG-3'	8-28 *E.c.*	1535	95° (94°, 55°, 72°) 72° (30)	III,	Weisburg et al., 1991
rD1 (pH')	5'-AAG GAG GTG ATC CAG CCG CA-3'	1542-1522 *E.c.*		3' (1', 1', 1') 3'	IV,V	
16S rRNA gene PCR for partial sequencing						
pA-B	5'-AGA GTT TGA TCC TGG CTC AG-3'	8-28 *E.c.*	1000	95° (94°, 55°, 72°) 72° (30)	III,	Edwards et al., 1989
pF'	5'-ACG AGC TGA CGA CAG CCA TG-3'	1073-1053 *E.c.*		3' (1', 1', 1') 3'	V	
pA	5'-AGA GTT TGA TCC TGG CTC AG-3'	8-28 *E.c.*	900	95° (94°, 55°, 72°) 72° (30)	IV	Edwards et al., 1989
pE'-B	5'-CCG TCA ATT CCT TTG AGT TT-3'	928-908 *E.c.*		3' (1', 1', 1') 3'		
amplification of catabolic genes						
*xylE*af	5'-TCG AGT TGC TGG GCC TGA TCG-3'	127-147 *P.p.*	731	94° (94°, 70°, 72°) 72° (30)	V	Jussila et al., 2006/
*xylE*ar	5'-CCC GCA GAA CAC TTC GTT GCG-3'	857-837 *P.p.*		10' (0.5', 1', 1') 3'		Paper V
*xylE*bf	5'-AGG TAT GGC GGC TGT GCG TTT C-3'	482-503 *P.p.*	469	94° (94°, 70°, 72°) 72° (30)	V	Jussila et al., 2006/
*xylE*br	5'-TTC GTT GAG AAT GCG GTC GTG G-3'	950-929 *P.p.*		10' (0.5', 1', 1') 3'		Paper V

Table 3-1 *(continued)*

C23O-ORF-F	5'-AGG TGW CGT SAT GAA MAA AGG-3'	-10-30 *P.p.*	934	95° (94°, 55°, 72°) 72° (35)	V	Junca & Pieper, 2003;
C23O-ORF-R	5'-TYA GGT SAK MAC GGT CAK GAA-3'	944-924 *P.p.*		5' (45'', 45'', 1.5') 8'		Junca & Pieper, 2004
16S rRNA gene PCR-DGGE (V3-region)						
PRBA338f (L_{GC})	5'-ACT CCT ACG GGA GGC AGC AG-3'	338-357 *E.c.*	236	94° (94°, 55°, 72°) 72° (30)	V	Lane, 1991;
PRUN518r (K)	5'-ATT ACC GCG GCT GCT GG-3'	534-518 *E.c.*		1' (0.5', 0.5', 1') 6'		Muyzer et al., 1993
GC clamp	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'					Muyzer et al., 1993

[a] f, forward primer; r, reverse primer;
-B, biotinylated primer;
L_{GC}, the GC clamp (40 nt) was attached to the 5' end of the forward primer L;

[b] A, Adenine; G, guanine; C, cytosine; T, thymine; I, inosine; U, Uracil; Y, pYrimidine (C or T); R, puRine (A or G); W, 'Weak' (A or T); S, 'Strong' (C or G); K, 'Keto' (T or G); M, aMino (C or A); D, not C; V, not T; H, not G; B, not A; X or N, unknown; O or -, deletion;

[c] *E.c.*, *Escherichia coli*; *P.p.*, *Pseudomonas putida* mt-2 (pWW0);

[d] PCR conditions are given in the following order: initial denaturation (denaturation, annealing, extension) final extension (cycles).

Table 3-2. Methods used for specific experimental purposes of the thesis.

Purpose/ Analysis	Method (equipment)	Described and used in paper no.				Reference(s)
Set-ups of plant tests						
in vitro	test tubes in growth chamber	I				Vincent, 1970
microcosms	pots in greenhouse	I	III		V	
mesocosms	lysimeters in field			IV	V	
surface sterilization of seeds	hypochloride	I	III	IV		Lipsanen & Lindström, 1988; Räsänen et al., 1991
inoculations	preparation of peat inoculants	I	III	IV		Elomestari Ltd, Juva, Finland
	bacterial inoculation of seeds and soil	I	III	IV		
gardening	fertilization, watering, thinning	I	III	IV		
Impact on plants						
seed germination (ecotoxicological test)	germination-%	I		IV		
survival of seedlings	survival-%	I				
viability of seedlings after restitution	viable/non-viable	I				
growth	number of growth leaves	I				
yield	dry weight (DW) of shoots (+70˚C)	I				
root formation	root length and branching	I	III			
root hair deformation and infection threads	bright-field microscopy after staining with methylene blue	I				Vasse & Truchet, 1984
nodulation	number of nodules	I	III			
Nitrogen fixation						
nitrogenase activity	acetylene reduction assay, GC	I	III			Lindström, 1984 a, b
Soil physical and chemical analysis						
soil type classification	particle-size analysis	I	III	IV		Elonen, 1971
dry weight	mg/g fresh soil			IV		
water holding capacity (WHC)	WHC-%			IV		
pH	$CaCl_2$ and H_2O method	I	III	IV		
conductance	10 x mS/cm	I	III			
organic C	% or mg/g DW	I	III	IV		
exchangeable nutrients	mg/l	I	III			Viljavuuspalvelu Ltd, Mikkeli, Finland
Toxics in soil						
metals	mg/kg DW		III			Neste, Corporate Technology, Analytical Research, Porvoo, Finland
m-toluate	GC-MS	I				
total hydrocarbons (THC)	gravimetric method (%)	I	III			SFS 3009, 1980
mineral oils and petrolether extractable compounds	gravimetric method (mg/kg)			IV		SFS 3009, 1980
Sampling of rhizosphere soil	earth drill (ø 1 cm)	I	III	IV		
Isolation, purification, classical characterization and cultivation of bacteria		I	III	IV	V	
Development of isolation media for rhizosphere bacteria			III	IV		
	preparation of rhizosphere extract		III	IV		
	preparation of soil extract		III			
	preparation of root extract			IV		Zaat et al., 1988; Suominen et al., 2003
Selective plating	*m*-toluate in various concentrations		III	IV		
Subculturing	1% Tween 80 (non-ionic detergent)		III	IV		
Classical characterization						
colony morphology	stereo microscopy		III	IV		
cell morphology	phase-contrast microscopy		III	IV		
cell wall structure	light microscopy after gram-staining		III	IV	V	

Table 3-2 *(continued)*

Enrichment cultures	incubation of soil with *m*-toluate				V		
Enumeration of culturable bacteria							
total plate counts	CFU/g DW			IV			
selective plate counts with *m*-toluate	CFU/g DW			IV			
Catabolic screening of bacteria							
m-Toluate tolerance test	maximal growth on agar	I	III	IV	V		
Utilization of *m*-toluate as the sole carbon source	maximal growth on agar		III	IV	V		
Indirect detection of aromatics-degrading/ TOL plasmids							
Catechol 2,3-dioxygenase (C23O) activity	catechol spray test		III	IV	V	Zukowski et al., 1983	
Antibiotics as selection markers							
Natural antibiotic resistance test	antibiotic sensitivity assay on agar				V		
Tagging of bacterial strains with antibiotic resistance	spontaneous mutation				V		
Horizontal gene transfer (HGT)/ plasmid transfer between bacteria							
Conjugations *in vitro*	filter-matings on agar				V		
in vivo	detection by strain isolation and plasmid characterization				V		
Stability test for plasmids	subculturing				V		
DNA extraction and purification							
reference strains	CTAB method		III	IV	V	Wilson, 1994	
rhizosphere bacteria	CTAB method or diatomaceous earth (Celite) method		III			Wilson, 1994; Boom et al., 1990; Heyd & Diehl, 1996	
	combined GTC-CTAB method			IV		Wilson, 1994; Chomczynski & Sachhi, 1996; Nick et al., 1999	
community DNA from rhizosphere	Fast DNA® SPIN Kit for Soil (BIO101) and freezing-thawing				V		
from denaturant gradient polyacrylamide gel (DGGE)	diffusion				V	Muyzer et al., 1993; Øvreås et al., 1997	
Separation of DNA fragments							
agarose gel electrophoresis (AGE)	DNA visualized under UV after EtBr staining		III	IV	V	Sambrook et al., 1989	
DNA quantification							
in AGE	comparison to DNA standards		III	IV	V	Sambrook et al., 1989	
spectrophotometrically	GeneQuant RNA/DNA Calculator				V		
Molecular typing/grouping of bacteria							
Genomic fingerprinting	rep-PCR with REP, ERIC and BOX primers		III			Versalovic et al., 1991, 1994; de Bruijn 1992; Nick et al., 1999	
	$(GTG)_5$-PCR		III	IV		Versalovic et al., 1991, 1994; de Bruijn 1992; Nick et al., 1999	
Taxonomic ribotyping	16S rRNA gene PCR-RFLP		III	IV	V	Weisburg et al., 1991; Laguerre et al., 1994	
DNA amplification by polymerase chain reaction (PCR)							
design of primers	PRIMER 0.5				V		
specificity tests of primers	temperature-gradient PCR				V		

(continued on next page)

Table 3-2 *(continued)*

Detection of plasmids by PCR					
xylE-PCR for TOL plasmids	gene-specific primers			V	
C23O-PCR for aromatics-degrading plasmids	degenerated, group-specific primers			V	Junca & Pieper, 2003, 2004
Profiling of degradation plasmids					
Amplified enzyme-coding DNA restriction analysis (AEDRA)	*xylE*-AEDRA			V	
	C230-AEDRA			V	
Molecular monitoring of changes in the function of bacterial community					
Enhanced TOL plasmid detection	nested C23O-*xylE*-AEDRA			V	
Molecular monitoring of changes in bacterial community structure					
Denaturant gradient gel electrophoresis (DGGE)	16S rRNA gene PCR-DGGE; primers for V3(and V1-V3, V6-V9) regions, SYBR® Gold staining			V	Muyzer et al., 1993; Øvreås et al., 1997
Molecular identification by DNA sequencing					
isolated bacterial strains	partial 16S rRNA gene	III	IV	V	Edwards et al., 1989; Institute of Biotechnology, University of Helsinki, Finland
non-isolated bacterial strains (DGGE fragments)	partial 16S rRNA gene			V	Høyteknologisenteret i Bergen, University of Bergen, Norway
oil-degradation genes	*xylE* and C23Os			V	Institute of Biotechnology, University of Helsinki, Finland
Statistical analyses/hypotheses testing					
one- and two-way analysis of variances (ANOVA)	Microsoft® Excel 2000	I	IV		

Table 3-3. Software used for bioinformatics in the thesis.

Computer program	Bioinformative purpose	Source and/or Reference	Paper
GelCompar 4.1	fingerprint analysis	Comparative Analysis of Electrophoresis Patterns; Applied Maths, Kortrijk, Belgium; www.applied-maths.com	III, IV
GCG package	sequence analysis	The GCG Wisconsin Package, Version 8.1-UNIX, August 1995; Genetics Computer Group, Madison, WI, USA; www.gcg.com (Accelrys GCG 11.0); usage through the Center for Scientific Computing (CSC), Espoo, Finland	III, IV
FastA	homology search and global sequence alignment	Pearson & Lipman, 1988	
BLASTn	homology search and local sequence alignment	Nucleotide Query Searching a Nucleotide Database; Altschul et al., 1990, 1997	
PileUp	multiple sequence alignment	Feng & Doolittle, 1987	
BLASTn 2	homology search and local sequence alignment	Basic Local Alignment Search Tool; National Center for Biotechnolgy Information (NCBI, USA); www.ncbi.nlm.nih.gov/blast; Altschul et al., 1990, 1997	III, V
ARB package	phylogenetic analysis	A Software Environment for Sequence Data; Munich, Germany; www.arb-home.de; Ludwig et al., 2004	III
PRIMER 0.5	primer selection	Computer Program for Automatically Selecting PCR Primers, May 1991; MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA, USA	V

4 RESULTS AND DISCUSSION

4.1 Potential of *Galega orientalis* for rhizoremediation of oil-contaminated soils (I)

4.1.1 Intrinsic *m*-toluate tolerance and degradation ablity of the model organisms

Axenic and *Rhizobium*-inoculated *Galega orientalis* plants were able to grow in the presence of 500 mg/l *m*-toluate. After paper I, we observed that *R. galegae* was able to tolerate even 3000 mg/l *m*-toluate after four days' cultivation on DEF8 medium which contains mannitol as the energy and carbon source. The inoculant *P. putida* PaW85 was well able to tolerate 9000 mg/l *m*-toluate. It grew best when 2000 to 7000 mg/l *m*-toluate was present. The optimal growth was detected in 6000 mg/l *m*-toluate. In contrast to *P. putida* PaW85, neither *G. orientalis* nor its microsymbiont *R. galegae* were able to degrade *m*-toluate on DEF8 agar without mannitol or in Jensen tubes.

4.1.2 Plant responses under *m*-toluate stress *in vitro*

The germination of *G. orientalis* seeds decreased with increasing *m*-toluate concentration illustrating the toxic effect of this aromatic compound. A *m*-toluate concentration higher than 500 mg/l inhibited *Rhizobium*-inoculated plants to grow and to develop roots. In general, the roots were stunted and branched when grown in *m*-toluate. No nodules formed, and the root hairs were deformed and swollen. No infection threads were present in the root hairs. In the highest *m*-toluate concentration tested (3000 mg/l), most of the plants were viable. When transferred into *m*-toluate-free medium, half of the plants began to grow normally and nodules developed on the new lateral roots within three weeks. The presence of *P. putida* PaW85 increased the tolerance level of the *Rhizobium*-inoculated plants up to 1000 mg/l via the degradation activity of *Pseudomonas* (Fig. 4-1). The root hairs of these plants were deformed and nodulation occurred normally.

4.1.3 Plant responses to oil-contamination in the greenhouse

Germination of *Galega* seeds was reduced by 10-14% in oil (3% THC) or *m*-toluate (2000 mg/l) contaminated soil. After four months, the dry matter yield of *G. orientalis* plants showed significant differences in growth between inoculant treatments but not between the soil types. The *Rhizobium*-inoculated pots gave significantly higher dry matter yields in all soil types. The presence of the *P. putida* PaW85 incoulant reduced the growth slightly. *Pseudomonas* inoculant was not necessary suitable for bioremediation because of this possible detrimental effect on the plant (Paper II). However, the bioaugmented rhizoremediation with *P. putida* PaW85 might be beneficial at the level of rhizosphere, which was studied later (ch. 4.4).

Rhizobium-inoculated plants nodulated normally in all soil types. The acetylene reduction assay showed that these plants were also able to fix nitrogen in all soil types. Root structure varied in different soil types. In oil soil the roots first spread mostly laterally, while *m*-toluate caused strong root branching similar to that observed *in vitro*. After the first reactions, the roots grew normally producing strong broadly branched root systems reaching into the contaminated soil layer. After four months, the *m*-toluate concentration in all pots decreased below the detectable level, and the roots filled all pots by the end of the experiment.

4.1.4 The symbiotic *Galega orientalis – Rhizobium galegae* system as a promising method for rhizoremediation of oil-contaminated soils

R. galegae was able to tolerate surprisingly high *m*-toluate concentrations. The growth and nodulation of *G. orientalis* were sensitive to *m*-toluate *in vitro* but in microcosms with oil- or *m*-toluate-contaminated soils plant growth and symbiotic functions were normal. The plant developed strong rhizosphere both in oil- and *m*-toluate-contaminated soils. In Jensen tubes in the presence of *P. putida* PaW85, the growth and development of root nodules was restituted while

the contaminant concentration in the media decreased as a result of *P. putida* PaW85 activity. In soils, inoculation with *P. putida* PaW85 reduced the plant growth slightly. The possibility that microbial communities in the rhizosphere are involved in the protection of plants from chemical injury has been an issue discussed in the literature of microbial degradation of xenobiotics (Anderson et al., 1993; Radwan et al., 1995). Excellent degraders *in vitro* are not necessary the best cooperation partners in natural environment (Kuiper et al., 2004). Most certainly, intrinsic degraders will arise from the near vicinity of plant roots to be better adapted to benefit also from aromatics. Taking into account the high aromatic resistance of *R. galegae* and the viability of *Galega* plant in oil-polluted soils, the symbiotic legume system seemed to be promising method for the rhizoremediation of oil-contaminated soils.

Fig. 4-1. *M*-toluate tolerance of *Galega orientalis in vitro*. Thirty-day-old seedlings were grown in various *m*-toluate concentrations (from left to right: 0, 500, 1000, 2000, and 3000 mg/l) in Jensen test tubes in the light chamber. Seedlings were inoculated with **A.** *Rhizobium galegae* HAMBI 1174, and **B.** *R. galegae* HAMBI 1174 and *Pseudomonas putida* PaW85/pWW0.

4.2 Genetic diversity of culturable, *m*-toluate tolerating bacteria in oil-contaminated rhizosphere of *Galega orientalis* (III)

4.2.1 Development of molecular typing methods for grouping of heterogeneous rhizosphere bacteria

4.2.1.1 16S rRNA gene ribotyping

A minimal set of three restriction endonucleases (*Alu*I, *Msp*I, *Rsa*I) was needed to detect simultaneously a wide variety of bacterial genera in 16S rRNA gene ribotyping. Seven gram-negative and six gram-positive ribotypes were revealed among 52 *m*-toluate tolerating bacteria from oil-contaminated *Galega* rhizosphere. Different sets of enzymes may be needed to resolve strains within certain species. Interestingly, *Alu*I alone could be used to separate different genera from each other. It could also, to some extent, differentiate strains even at species level inside the genera *Ralstonia* and *Pseudomonas*. On the other hand, gram-negative and gram-positive bacteria were slightly intermingled in computer-assisted analysis based on only *Alu*I.

4.2.1.2 Genomic fingerprinting with $(GTG)_5$ primers

$(GTG)_5$-PCR was the most useful rep-PCR method to group heterogeneous rhizosphere bacteria. At the similarity level of 70 %, gram-negative and gram-positive strains were clustered into 11 and 12 $(GTG)_5$-groups, respectively. The groups represented different species. Species in the same genera were grouped together but the location of the genera did not necessarily reflect their phylogenetic relationships. Only *Pseudomonas* and *Bacillus* species were mostly interspersed through the dendrograms demonstrating their heterogeneous genomic nature. In addition, *Ralstonia eutropha* was divided into two $(GTG)_5$-genotypes, though grouped with 0.62 similarity, and the *Rhodococcus/Nocardia* branch into four $(GTG)_5$-genotypes, which formed a group at the 0.39 similarity level.

Despite the careful standardization at all levels, the fingerprints even from the same strain may vary so much that the similarity percents in dendrograms cannot be taken as absolute identity measures. Instead, in this study it was shown that $(GTG)_5$-PCR genomic fingerprinting could also be used to infer the degree of shifts in the community structure at species level. These results are supported by the observation that the genomic structure of a bacterium, as deduced from its genomic fingerprint, represents an accurate reflection of its taxonomic and phylogenetic position based on total genomic DNA-DNA hybridization values (Rademarker et al., 2000). There are only two previous reports on the use of the $(GTG)_5$ primer for genomic fingerprinting: one on gram-negative rhizobia (Nick et al., 1999) and another on gram-positive *Lactobacillus* (Gevers et al., 2001). Based on the present study, $(GTG)_5$ fingerprinting is recommend for both gram-negative and gram-positive bacteria, though in separate analysis.

4.2.1.3 Molecular toolbox for studying genetic diversity of culturable rhizosphere bacteria in oil-contaminated soil

$(GTG)_5$-genotyping proved to be a powerful method differentiating bacterial species. At the same time, it separated even different strains among *Pseudomonas oryzihabitans* and *Ralstonia eutropha*. It also revealed some intricacies in the naming of *Rhodococcus/Nocardia* and *Bacillus* species like Gürtler et al. (2004) had noticed based on other methods. Similarly, the heterogeneous genomic nature of *Pseudomonas* was revealed. Generally, the $(GTG)_5$-genotype and the 16S-ribotype (or subtype) corresponded to each other very well representing a species. In some cases, 16S-ribosubgrouping revealed more differences inside species than $(GTG)_5$-genotyping, like in the case of *Arthrobacter histidinolovorans*. On the other hand, the restriction endonucleases used might not be the best ones for all the genera (*Rhodococcus/Nocardia*). Thus,

the information obtained from the single locus ribotyping and $(GTG)_5$-genotyping complemented each other. When combined with selective partial sequencing of 16S rRNA genes, they offered rapidly gainable and relevant phylogenetic information to be used in microbial ecology studies of rhizosphere populations.

4.2.2 Identification and phylogenetic analysis of the isolated bacterial strains from oily rhizosphere

High phylogenetic diversity was observed in the form of five major lineages of the Bacteria domain: α-, β- and γ-Proteobacteria, and gram-positives with both high and low G+C%. Gram-positive *Rhodococcus*, *Bacillus* and *Arthrobacter* and gram-negative *Pseudomonas* were the most abundant genera. In the oil-contaminated rhizosphere, gram-positive bacteria dominated, while the best *m*-toluate degraders were found among the genus *Pseudomonas*. The dominance of either gram-positive or gram-negative bacteria in contaminated soil might depend on the toxic compound and on the level of its toxicity (Sandaa et al., 1999b; Wagner-Döbler et al., 1998).

4.3 *M*-toluate tolerance and degradation capacity of the isolated rhizosphere bacteria

Rhizosphere bacteria were isolated from the greenhouse (microcosms) (Fig. 4-2) and field lysimeter (mesocosms) (Fig. 4-3) experiments.

4.3.1 Greenhouse experiment (III)

Only one-fifth of the strains that tolerated *m*-toluate also degraded *m*-toluate. Some of the tolerant strains might be involved in the degradation of other hydrocarbons present in the oil-contaminated soil, probably also in co-metabolising. The ability to degrade *m*-toluate by a TOL plasmid was detected only in species of the genus *Pseudomonas*. Only one strain, *Pseudomonas oryzihabitans* 29 (H2397), was catechol positive and degraded *m*-toluate as efficiently as the inoculant *P. putida* PaW85 (H1828), which was not found in the rhizosphere samples. The reduction of the catechol 2,3-dioxygenase (C23O) activity in the other strains might indicate that the cell did not completely support TOL functions after the reception of a TOL plasmid. On the other hand, various TOL plasmids can have different C23O activity levels. Strain-specific differences in degradation abilities were found for *P. oryzihabitans*. In addition, *Pseudomonas migulae* strains and a few *P. oryzihabitans* strains were able to grow on *m*-toluate and most likely contained a TOL plasmid. These findings were new and had not been reported before this study.

The best *m*-toluate degraders isolated in this study belonged to the genus *Pseudomonas*. Most solvent-tolerant strains isolated have been found to belong to the genus *Pseudomonas* (Isken & de Bont, 1998). Other genera, such as *Bacillus* (Abe et al., 1995) and *Rhodococcus* (Andreoni et al., 2000), have also been shown to include solvent-tolerant strains. Several catechol 2,3-dioxygenases have been found from different *Rhodococcus* species and strains (Candidus et al., 1994; Kosono et al., 1997; Kulakov et al., 1998; Taguchi et al., 2004). However, according to the nucleotide databases, the C23O genes of *Rhodococcus* were not closely related to the ones of *Pseudomonas*. We showed that several gram-positive strains representing the genera *Rhodococcus*, *Arthrobacter*, *Nocardia* and *Bacillus* tolerated very high amounts of *m*-toluate. Some *Rhodococcus erythropolis* strains could even degrade *m*-toluate but not via the *meta*-pathway in the TOL plasmid. Thus, various intrinsic degradation potential existed in the oil-contaminated rhizosphere.

4.3.2 Field lysimeter experiment (IV)

Only 10% of the isolated, *m*-toluate tolerating strains were able to degrade *m*-toluate. Most of the *m*-toluate utilizing bacteria had the degradation pathway where C23O was involved indicating the presence of a TOL plasmid. However, the breakdown of *m*-toluate by gram-negative bacteria was not restricted to the TOL pathway. The others and also the gram-positive degraders may have

another degradation pathway encoded in a plasmid or on the chromosome. In addition, only part of the catechol positive degraders could use *m*-toluate as the sole carbon source indicating the presence of a different kind of substrate specificity.

The amount of heavy tolerants might have favoured the higher proportion of *m*-toluate tolerating bacteria in untreated and *Galega*-plus-*Pseudomonas* soils by transferring *m*-toluate degradation plasmids. When isolates were grown on TY agar and thereafter transferred again on TY-plus-*m*-toluate media, most of the isolates did not tolerate as high concentrations of *m*-toluate as before. This may be due to the loss of a plasmid or parts of it containing the tolerance and/or degradation genes (Bayley et al., 1977).

Most *m*-toluate degrading and catechol positive bacteria were found in soils where *Galega* plant was growing, which may indicate an increased efficiency in oil biodegradation. For example Sayler et al. (1985) have shown a correlation between the enhanced rates of PAH mineralization in oil-contaminated sediments and an increase in the number of colonies containing DNA sequences which hybridize to TOL (toluate oxidation) and NAH (naphthalene oxidation) plasmid probes using the colony hybridization technique. In our study, however, the variation between the bacterial counts obtained from different dilution series was high. Still, Palmroth et al. (2005) recently showed that the utilization of diesel fuel by soil bacteria was higher in contaminated soil, especially when vegetated, than in uncontaminated soil. In addition, diesel fuel disappeared more rapidly in the legume mixture treatment (white clover, *Trifolium repens* and pea, *Pisum sativum*) than in other plant treatments (Palmroth et al., 2002).

4.4 Rhizosphere effect of *Galega orientalis* in oil-contaminated soil (IV)

The potential rhizosphere effect (bacterial abundance and community composition) of *Galega orientalis* was explored in the field experiment. The hypotheses were that 1) the legume plant increases bacterial numbers and diversity in oil-contaminated rhizosphere and 2) bioaugmentation with conjugative *Pseudomonas putida* PaW85 increases bacterial diversity of *m*-toluate utilizing bacteria in *Galega* rhizosphere.

4.4.1 The effect of *Galega* rhizosphere and *Pseudomonas* bioaugmentation on the bacterial abundance

Galega orientalis but also *Pseudomonas putida* increased the overall number of bacteria in the field experiment with oil-contaminated soil. *G. orientalis* especially together with *Pseudomonas* bioaugmentation, increased the amount of *m*-toluate tolerants, which may indicate an increased

Fig. 4-2. The greenhouse experiment.

Fig. 4-3. The field lysimeter experiment.

efficiency in oil degradation under these favourable rhizosphere conditions. In previous studies, too, plant rhizosphere has been found to increase the bacterial concentrations (Pilon-Smits, 2005; Salt et al., 1998; Atlas & Bartha, 1993; Lee & Banks, 1993). However, the observed effects of bioaugmentation have been contradictory (Vogel, 1996). In this study, *P. putida* PaW85 may have increased the bacterial numbers by providing a conjugative plasmid, which helps other bacteria to adapt to the oil-contaminated environment. The assessment of the benefits of *P. putida* bioaugmentation is complicated by many factors that also affect the bioremediation efficiency. Significant factors may be physical and chemical properties of the soil, the properties of the soil contaminants (bioavailability, concentration and toxicity), microbial ecology (predation and competition), and the way in which the bioaugmentation is done (Vogel, 1996).

4.4.2 The effect of *Galega* rhizosphere and *Pseudomonas* bioaugmentation on the bacterial community composition

Densities of rhizosphere bacteria can be two to four order of magnitude greater than in the surrounding bulk soil (Pilon-Smits, 2005; Salt et al., 1998). According to Killham (1994) the microbial diversity in the rhizosphere can also be lower than in the bulk soil. In the present study, the rhizosphere of *G. orientalis* did not reduce the diversity of bacteria, since more different ribotypes were found in the *G. orientalis* containing soils compared to the untreated soil. The rhizosphere of *G. orientalis* seemed to increase the diversity of bacteria. However, the diversity of the *m*-toluate degrading bacteria did not significantly increase. The number of different ribotypes was highest in the bioaugmented soil and lowest in the untreated soil. In bioaugmented soils, also gram-positive ribotypes were observed. This might indicate a positive effect of *Pseudomonas* bioaugmentation to also activate gram-positive degraders. Notable is that *P. putida* PaW85 did not form any superior population in the soils.

Both gram-negative and gram-positive bacteria play important roles in oil degradation. In this study, the phylogenetically identified strains *P. migulae* and *A. aurescens* were able to degrade *m*-toluate. *Pseudomonas* and *Arthrobacter* are genera commonly found in oil-contaminated soils (Atlas, 1981). Representatives of both genera are also capable of producing plant stimulating organic compounds (Atlas & Bartha, 1993). The communication, i.e. signal exchange, between tolerant soil bacteria and plant might explain partly the favourable conditions prevailing in oil-contaminated *Galega* rhizosphere.

An interesting finding was that only half of the strains representing *P. migulae* ribotype were catechol positive and able to degrade *m*-toluate as the sole carbon source and thus, most likely contained the TOL plasmid. The horizontal transfer of degradative plasmids could play an important role for rhizosphere bacteria to survive well in oil-contaminated soils (Sarand et al., 2000).

4.4.3 Three aspects of the rhizosphere effect

The rhizosphere effect of *Galega orientalis* was manifested in oil-contaminated soil. The first hypothesis was supported, since *G. orientalis* increased not only the total bacterial numbers but also the numbers of *m*-toluate utilizing and catechol positive bacteria indicating an increase in degradation potential. Also the bacterial diversity, when measured as the amount of ribotypes, was increased in *Galega* rhizosphere. However, the diversity of *m*-toluate utilizing bacteria did not significantly increase. On the contrary, the second hypothesis was not supported. The bioaugmentation with conjugative *P. putida* PaW85 did not significantly increase the diversity of *m*-toluate utilizing bacteria in *Galega* rhizosphere. However, *Pseudomonas* increased the overall bacterial diversity, especially the amount of gram-positive ribotypes. *Pseudomonas* bioaugmentation could also increase bacterial numbers and especially together with *Galega* plant the amount of *m*-toluate utilizing bacteria, which might have been triggered by conjugation. In addition to bacterial numbers and diversity, a third sign of the rhizosphere effect was the fact that

a part of *P. migulae* strains (and *P. oryzihabitans* strains) were able to degrade *m*-toluate as the sole carbon source. This indicated that (a) conjugative plasmid(s) exists among the rhizosphere bacteria. Taken all together, rhizoremediation seems to work in such a way that the legume stimulates bacterial proliferation and *m*-toluate degradation, which may facilitate, in turn, roots to grow deeper into the contaminated soil.

4.5 TOL plasmid transfer during bacterial conjugation *in vitro* and rhizoremediation of oil compounds *in vivo* (V)

4.5.1 Detection of TOL plasmids by the designed gene-specific *xylE*-PCR

The gene-specific detection method, *xylE*-PCR, was designed in this work to detect TOL plasmids (e.g. pWW0 and pWW53) (Fig. 4-4). It was important to verify in practice the strict annealing conditions needed for *xylE*-PCR to avoid false positives among the evolutionary similar genes like *nahH* (Ghosal et al., 1987). The gene *xylE* encoding one of the key enzymes, catechol 2,3-dioxygenase, within the *meta*-pathway on the TOL plasmid pWW0 was chosen as the target of the specific PCR reaction because catechol is one of the few key biochemical intermediates to dissimilate a variety of aromatic compounds into the Krebs cycle (Assinder & Williams, 1990; Houghton & Shanley, 1994). The catechol 2,3-dioxygenase and thus, the presence of a TOL plasmid, was also determined indirectly through the visible end product in the catechol spray test. Thus, we were able to detect both potential and active *m*-toluate degraders. In addition, the *xylE*-PCR was indispensable to detect the catechol 2,3-dioxygenase from yellow-pigmented bacteria like *Arthrobacter* and *Sphingomonas*.

P. aeruginosa JI104, plasmids of which are undetermined, seemed to harbor a TOL plasmid. In addition, a plasmid-borne *phlH* gene was found from the reference strain *P. putida* ATCC 11172 with degenerative C23O primers. According to Molin & Nilsson (1985), *P. putida* ATCC 11172 has an uncharacterized *meta*-cleavage enzyme for phenol degradation. A new finding in this study was that *P. putida* ATCC 11172 harbored a C23O gene identical to *phlH* of *P. putida* H and, thus, most probably contained a PHE plasmid. *P. putida* H is a (methyl)phenol/cresols degrader harboring a 220-kb and conjugative PHE plasmid pPGH1, on which the *phlH* gene is situated (Herrmann et al., 1987, 1988, 1995; Lauf et al., 1998).

4.5.2 Monitoring horizontal plasmid transfer

4.5.2.1 *In vitro* transfer of a TOL plasmid from *Pseudomonas putida* to *Rhizobium galegae*; Development of *Rhizobium* inoculants with dual function

Sinorhizobium meliloti Orange 1 was the first reported nodule-forming strain capable of degrading dibenzothiophene (DBT) (Frassinetti et al., 1998). It was the first example of a symbiotic *Rhizobium*, which has a metabolic pathway mechanism analogous to that described for naphthalene degradation in other bacteria. This suggested the potential use of rhizobia inoculants for bioremediation connected to a development of strains provided with dual functions: degradation of contaminants and nitrogen fixation. In the present work, the conjugation of TOL plasmid from *Pseudomonas* (γ-Proteobacterium) into *Rhizobium* (α-Proteobacterium) was complicated because, firstly, *Rhizobium* produced exopolysaccharide that can function as a physical barrier against the transfer process. Secondly, the donor was able to survive in this slime despite the selection against it in matings. In addition, the *Rhizobium* transconjugants harboring the TOL plasmid seemed to be unstable. This instability may arise from the location of TOL catabolic genes in a 59-kb transposon (Assinder & Williams, 1990). Similarly, the TOL plasmid seemed not to be stably maintained in *Erwinia chrysanthemi* (Ramos-Gonzalez et al., 1991). The environmental pressure simulated by the mating selection with *m*-toluate did not help to obtain *Rhizobium* transconjugants. Similarly, Pinedo & Smets (2005) found no positive effect on the TOL plasmid transfer frequency by premating exposure to ethanol, toluene or phenol. Also the

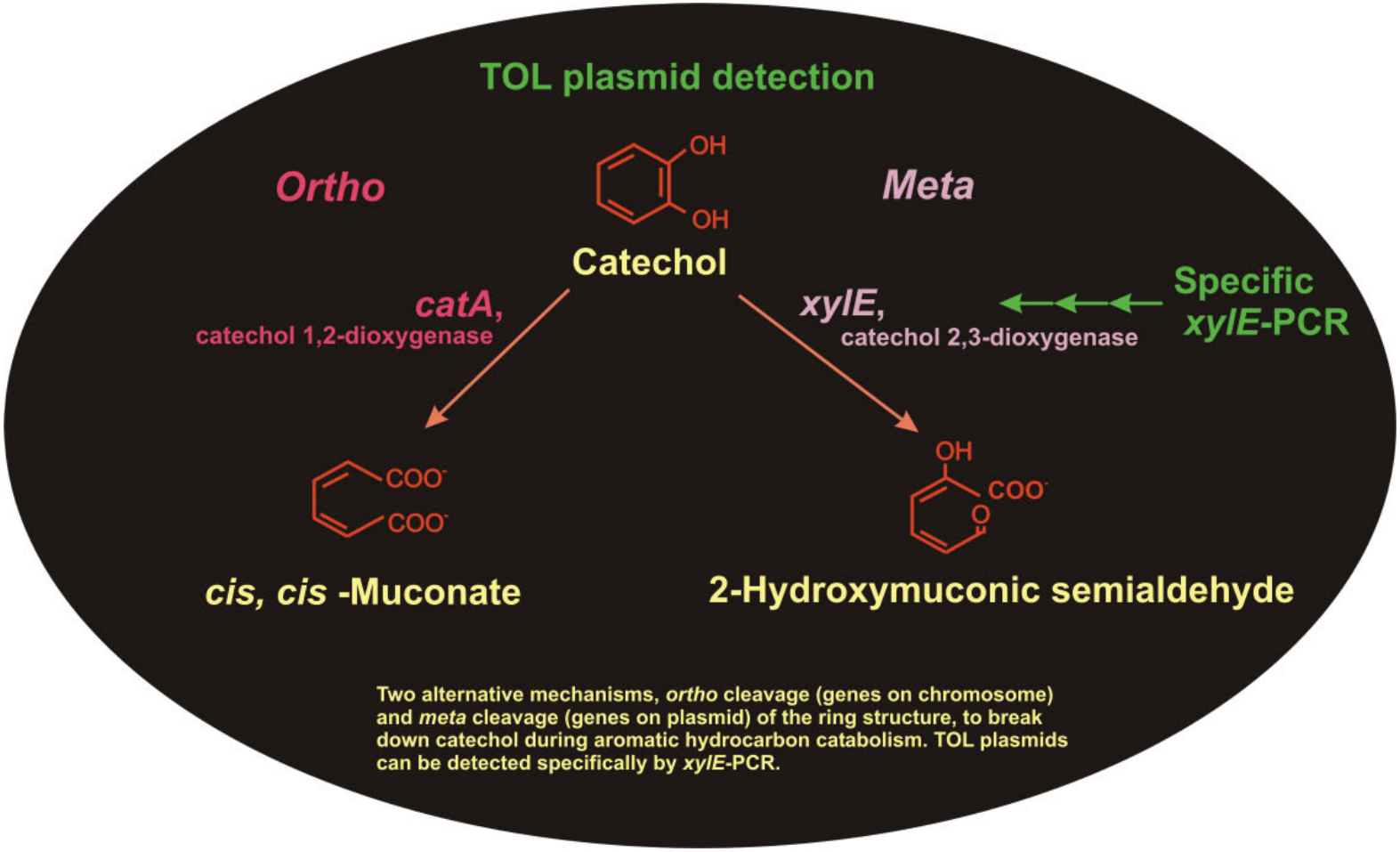


Fig. 4-4. The basis of the gene-specific *xylE*-PCR designed to detect TOL plasmids.

available *in situ* data of stimulatory effects of stresses on conjugation rates has been ambiguous (Sørensen et al., 2005).

Ramos-Gonzalez et al. (1991) did not observe any transfer of the TOL plasmid to *Rhizobium meliloti*, *Acinetobacter calcoaceticus* or *Alcaligenes* sp. In the present study, 50% of the conjugant candidates were transconjugants originally but only 12% of the candidates were stable. These represented *Rhizobium galegae* bv. *officinalis*, not the symbiont *R. galegae* bv. *orientalis* of our model plant *Galega orientalis*. It is unknow yet, whether the whole TOL plasmid had conjugated from *Pseudomonas* to *Rhizobium* or a few degradation genes had integrated into the *Rhizobium's* chromosome by transposition. The second possibility is supported by the fact that the transconjugants did not grow with the antibiotics selective for the TOL plasmid and that they were stable. However, the presence of the *xylE* gene did not increase the capability of *Rhizobium* to degrade *m*-toluate. Thus, no such *Rhizobium* transconjugants were produced, which would have degraded oil and, thus, helped in that way the bioremediation process in rhizosphere with *G. orientalis*. However, even non-degrading transconjugants may serve as a plasmid-reservoir, which might facilitate the adaptation of the community upon encountering the corresponding xenobiotic compound (Bathe et al., 2004). Additionally, they may permit transfer of the plasmid to recipients that were not accessible by the initial donor strain.

4.5.2.2 Identification of aromatics-degrading plasmids among the bacterial strains isolated from the oil-contaminated rhizosphere of *Galega orientalis*

The observed effects of bioaugmentation have been contradictory (Vogel, 1996). But an increase in the frequency of catabolic plasmids within a community has often been observed in pollutant-stressed environments (Sayler et al., 1990). In the present study, a few new *m*-toluate degrading bacteria were characterized: pDK1-type TOL plasmids have not been found from *Pseudomonas migulae* or *P. oryzihabitans* before. We also verified that *P. oryzihabitans* 29 harbored the pWW0 plasmid that most probably had conjugated from the bioaugmentation bacterium *P. putida* PaW85. Even if there are no ecological niches available for the bioaugmentation bacterium itself, its conjugative catabolic plasmid or genes can have some additional value for other bacterial species and for bioremediation. In addition, a pDK1-type TOL plasmid improved the ability of *P. migulae* (26a) to degrade *m*-toluate in comparison with a pDK1-type TOL plasmid in *P. oryzihabitans* (19a, 27b) or in the pDK1 in *P. putida* HS1. Thus, the reception of different catabolic plasmids seemed to be a method for intrinsic rhizosphere bacteria to increase their potential to survive in oil-polluted soil.

Plasmids pWW0 and pDK1 have been speculated to be the exceptions rather than the rule in carrying only a single set of *meta*-pathway genes (Assinder & Williams, 1990). Many independently isolated TOL plasmids from geographically diverse locations have carried two genes for C23O. For instance, the majority of the plasmids isolated from oil-contaminated soil samples near Minsk, Belarus carried *xyl*-type genes highly homologous to those of pWW53 and organized in a similar manner with two distinguishable *meta*-pathway operons (Sentchilo et al., 2000). In addition, opposite to the generally observed incompatibility rules, plasmids can also enter cells that carry a closely related element (Thomas & Nielsen, 2005). According to the background observed during sequencing of the C23O gene of the pDK1-type TOL plasmids, *P. oryzihabitans* 27b, 19a and *P. migulae* 26a either carry two operons for the C23O gene (one homologue to the archetypal *xylE* gene) or they harbor a pWW0-type TOL plasmid in addition to their pDK1-type TOL plasmid. Plasmid isolation, cloning and sequencing would enlighten the situation.

XylE-AEDRA was useful to genetically track one class of degradation plasmids, TOL plasmids. In turn, C23O-AEDRA was a suitable method to profile various C23O containing catabolic plasmids. Since bacterial strains were selectively isolated from oil-contaminated rhizosphere with variable concentrations of *m*-toluate, it was no surprise that all the catechol-positive *m*-toluate degraders harbored a TOL plasmid. On the other hand, other C23O containing bacteria could also have been detected because they were able to degrade *m*-toluate to some extent. Thus, this study showed that TOL plasmids were a major group of degradation plasmids in the oil-contaminated rhizosphere of *Galega orientalis*. Still, *nahAc*- and *alkB*-PCR revealed

that the potential for degradation of also other oil compounds exists in our rhizosphere isolates (Paper II).

4.5.3 Molecular monitoring of changes through direct DNA analysis during bioremediation in the *m*-toluate contaminated rhizosphere of *Galega orientalis*

4.5.3.1 Diversity of TOL plasmids

The plasmid pWW0 could only be detected at the beginning of the experiment in the treatments, in which the inoculant *P. putida* PaW85 was added (P, GP). At the beginning of the experiment, a pDK1-type TOL plasmid could already be detected only in *Galega*-plus-*Pseudomonas* treatment. Throughout the rhizoremediation experiment, a pDK1-type TOL plasmid similar to the one found from the isolated *P. oryzihabitans* 27b could be detected in all the samples indicating the presence of effective intrinsic degraders to exist in the soil. However, like with isolates, the possible existence of an archetypal/pWW0-type TOL plasmid could not be excluded due to overlapping patterning in AEDRA analysis. Alternatively, two homologous *xylE* genes, one of which represents the archetypal *xylE* gene, might exist in these pDK1-type TOL plasmids.

Rhizosphere effect of *G. orientalis* was manifested during rhizoremediation experiment by increasing the numbers of *m*-toluate utilizing and catechol-positive bacteria especially together with *Pseudomonas* bioaugmentation (Paper IV). This indicated an increase in degradation potential. Tuomi et al. (2004) observed, in turn, that the abundance of *nahAc* genes correlated with the ^{14}C-naphthalene mineralization potential in petroleum hydrocarbon-contaminated soils. At the end of our experiment, no TOL plasmid could anymore be detected in the soil treated with both *G. orientalis* and *P. putida* PaW85. Most probably the detection limit for TOL plasmids was encountered indicating decreased amount of degradation plasmids and, thus, the success of rhizoremediation.

4.5.3.2 Bacterial community profiling

Diversity is composed of two elements, richness and evenness. The highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994; Kapur & Jain, 2004). To assess effects of different treatments (vegetation, bioaugmentation) under selective pressure (*m*-toluate contamination) on bacterial community diversity, the richness and evenness of bacteria was detected according to the number and thickness of DGGE bands, respectively. The strongest DGGE bands were supposed to represent numerically dominant bacteria. However, it is possible that several tightly packed DGGE bands can be observed as one strong DGGE band. In addition, some bands could be strong because the 16S rRNA gene sequences they represent might be easier to amplify than some other bands. With these limitations in mind, 16S rRNA gene PCR-DGGE was used to give an overview of the bacterial diversity.

Palmroth et al. (2005) showed that the utilization of diesel fuel by soil bacteria was especially high in vegetated, contaminated soil. In the present work, smear-like and tight patterning in DGGE gels most probably indicated high richness of bacterial species in vegetated soils. In addition, less dominant bacteria and, thus, more even bacterial composition was observed in soils treated with *G. orientalis* compared to non-vegetated soils. More even distribution of bacteria and thus, diversified communities may best guarantee the overall success in rhizoremediation by offering various genetic machineries for catabolic processes. Different bacteria seemed to be favoured in soils treated with either *G. orientalis* or *Pseudomonas*. This may indicate different mutual benefits in these different co-operational contexts. *Pseudomonas* bioaugmentation seemed to minimize changes in the diversity of the bacterial community both in non-vegetated and vegetated soil. However, the bioaugmentation bacterium *P. putida* PaW85 did not seem to be a dominant bacterium. In turn, the *Galega* plant seemed to minimize changes in the bacterial dominance. These observations may indicate that both *Pseudomonas* and the *Galega* plant have their influence to maintain certain populations in soil.

It is well known that *Pseudomonas* play an important role in degradation processes of *m*-toluate (Houghton & Shanley, 1994). Indeed, three dominant bands sequenced represented different species of *Pseudomonas*. *P. migulae*, representing one dominant species in the contaminated soil community according to DGGE gels, was also isolated and turned out to be an excellent and a new type of degrader of *m*-toluate.

4.5.4 Applications for environmental biotechnology

In the era of direct DNA analysis we cannot forget the importance of the isolation and characterization of bacteria and their catabolic plasmids. The findings in the Paper V suggest that the intrinsic biodegradation potential of oil-polluted soil and the success of bioremediation could be estimated by monitoring changes not only in the type and amount but also in transfer of degradation plasmids and thus, as increased functional diversity within bacterial populations.

5 CONCLUSIONS

The following conclusions summarize the main findings of this work in relation to the original, specific aims when studying **the microbiology of rhizoremediation**:

1. *Galega orientalis* is a perennial plant, it has a large root system, and its cultivation practice is optimized. All these characters are beneficial for a plant to be used in rhizoremediation. Based on the high aromatic tolerance of *Rhizobium galegae* and the viability of *Galega* plants in oil-polluted soils, the symbiotic legume system proved to be promising method for the rhizoremediation of oil-contaminated soils. The use of *G. orientalis* for rhizoremediation purpose is unique in the world.

2. Molecular biomonitoring methods were designed and/or developed further for bacteria and their degradation genes. A molecular toolbox consisting of genomic fingerprinting with $(GTG)_5$ primers, taxonomic ribotyping of 16S rRNA genes and selective partial 16S rRNA gene sequencing was developed for grouping of culturable, heterogeneous rhizosphere bacteria even at species level. In addition, PCR primers specific for the *xylE* gene were designed for TOL plasmid detection. The sensitivity of the direct monitoring of TOL plasmids in soil was enhanced by nested C23O-*xylE*-PCR. AEDRA analysis with one restriction enzyme (*Alu*I) was used to profile both TOL plasmids (*xylE* primers) and, in general, aromatics-degrading plasmids (C230 primers).

3. a) A culture collection of indigenous *m*-toluate tolerating bacteria from oil-contaminated rhizosphere of *Galega orientalis* was established. These bacteria serve as reference strains for molecular biomonitoring of culturable rhizosphere bacteria, individuals or consortia, and a reservoir to study further different tolerance and degradation mechanisms for aromatics. **b)** High (phylo)genetic diversity was observed among the isolated, *m*-toluate tolerating rhizosphere bacteria in the form of five major lineages of the Bacteria domain. Gram-positive *Rhodococcus*, *Bacillus* and *Arthrobacter*, and gram-negative *Pseudomonas* were the most abundant genera. **c)** The inoculum *Pseudomonas putida* PaW85/pWW0 was not found in the rhizosphere samples. Even if there were no ecological niches available for the bioaugmentation bacterium itself, its conjugative catabolic plasmid might have had some additional value for other bacterial species and thus, for rhizoremediation.

4. Only 10 to 20% of the isolated, *m*-toluate tolerating bacterial strains were also able to degrade *m*-toluate. The ability to degrade *m*-toluate by using enzymes encoded by a TOL plasmid was detected only in species of the genus *Pseudomonas*, and the best *m*-toluate degraders were these *Pseudomonas* species. Strain-specific differences in degradation abilities were found for *P. oryzihabitans* and *P. migulae*: some of these strains harbored a TOL plasmid – a new finding observed in this work, indicating putative horizontal plasmid transfer in the rhizosphere. The breakdown of *m*-toluate by gram-negative bacteria was not restricted to the TOL pathway. In addition, some catechol positive strains were not able to use *m*-toluate as the sole carbon source indicating the presence of other aromatics-degrading plasmids. Several gram-positive genera tolerated high amounts of *m*-toluate. Some *Rhodococcus erythropolis* and *Arthrobacter aurescens* strains could even degrade *m*-toluate in the absence of a TOL plasmid.

5. Rhizosphere effect of *G. orientalis* was manifested in oil-contaminated soil. *G. orientalis* and *Pseudomonas* bioaugmentation increased the **amount of rhizosphere bacteria**. *G. orientalis* especially together with *Pseudomonas* bioaugmentation increased the numbers of *m*-toluate utilizing and catechol positive bacteria indicating an increase in degradation potential. Also the **bacterial diversity**, when measured as the amount of ribotypes, was increased in the *Galega* rhizosphere with or without *Pseudomonas* bioaugmentation. However, the diversity of *m*-toluate utilizing bacteria did not significantly increase.

6. a) The archetypal TOL plasmid pWW0 was conjugated *in vitro* from *P. putida* to *R. galegae*. The *xylE* gene was detected both in *R. galegae* bv. *officinalis* and bv. *orientalis*, but it was neither stably maintained in *R. galegae* bv. *orientalis* nor functional in *R. galegae* bv. *officinalis*. Thus, no *Rhizobium* transconjugant with BTEX degradation genes was produced. **b)** TOL plasmids were a major group of catabolic plasmids among the rhizosphere bacteria. The following two observations might have indicated **horizontal TOL plasmid transfer** between rhizosphere bacteria and thus, the gene dynamics during rhizoremediation. One *P. oryzihabitans* strain harbored the pWW0 plasmid that had probably conjugated from the bioaugmentation bacterium *P. putida* PaW85. In addition, some *P. migulae* and *P. oryzihabitans* strains seemed to harbor both the pWW0- and the pDK1-type TOL plasmid. Alternatively, they might have harbored a TOL plasmid with both the pWW0- and the pDK1-type *xylE* gene.

7. Changes in the diversity of TOL plasmids and bacteria were monitored through direct DNA analysis. **a)** At the end of the experiment, no TOL plasmid could be detected in soil treated with both *G. orientalis* and *P. putida* PaW85. The detection limit for TOL plasmids was encountered indicating decreased amount of degradation plasmids and thus, the success of rhizoremediation. **b)** By using the 16S rRNA gene PCR-DGGE method, the highest richness of species was observed in vegetated soils and the distribution of bacteria was more even, i.e. more equal abundance of species observed, in vegetated soils compared with non-vegetated soils, illustrating once again the rhizosphere effect of *G. orientalis*. Diversified communities may best guarantee the overall success in rhizoremediation by offering various genetic machineries for catabolic processes. Both *Pseudomonas* bioaugmentation and the *Galega* plant seemed to minimize changes in the diversity of the bacterial community; diversity in the soils without the plants or *Pseudomonas* fluctuated more over time.

6 FUTURE PROSPECTS

Molecular biomonitoring methods for both the bacteria (amount and community composition) and their degradation genes (type, amount, expression, and transfer) are of great value in the way understanding molecular microbial ecology behind bioremediation processes and thus, forming the basis for environmental applications. The molecular biomonitoring methods could be applied for several purposes within the environmental biotechnology: to make a diagnosis of the status of contaminated soil, to map the genetic machinery of intrinsic microbes for degradation, to evaluate the intrinsic biodegradation potential and to predict the development of the contaminated soil; to be able to control and enhance the bioremediation process and; to estimate and monitor the success of bioremediation. In the long run, the environmental protection by using nature's own resources and thus, acting according to the principle of sustainable development, would be both economically and environmentally beneficial for society.

Microbial ecology studies the interactions between microorganisms and environment. When encountering recalcitrant compounds, bacteria with different degradation capabilities would most certainly benefit from co-operation. Therefore, the study of bacterial consortia as active bioremediation units and as possible inoculants is also important in the future. In addition, there is too little information on plasmid dynamics in soil to estimate the significance of horizontal gene transfer for the efficiency of bioremediation. Even systematic characterization of plasmids from natural isolates has largely been neglected thus far. Also the stimulatory effect of stress factors, e.g. oil compounds, on horizontal gene transfer *in situ* remains still ambiguous. Therefore, as the next step to understand more about the molecular microbial ecology behind bioremediation, we should pay attention to and study the whole **mobilome** (mobile gene pool, consisting of e.g. plasmids and transposons) in oil-contaminated soil.

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